Studies on crystalline yeast phosphoglucomutase(Dissertation_全文)

AUTHOR(S):

Hirose, Masaaki

STUDIES ON CRYSTALLINE YEAST PHOSPHOGLUCOMUTASE

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Abbreviations

Gl-P	α-D-glucose 1-phosphate
G6-P	D-glucose 6-phosphate
Gl,6-diP	α-D-glucose 1,6-diphosphate
Fl-P	D-fructose 1-phosphate
F6-P	D-fructose 6-phosphate
Fl,6-diP	D-fructose 1,6-diphosphate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PCMB	p-Chloromercuribenzoate
NBS	N-Bromosuccimide
ANS	1-Anilino-8-naphthalene sulfonate
EDTA	Ethylenediaminetetraacetate
Tris	Tris(hydroxymethyl)aminomethane

Introduction

Phosphoglucomutase (α-D-glucose 1,6-diphosphate : α-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) catalyzes the reversible interconversion between Gl-P and G6-P, and requires Gl,6-diP as a coenzyme[1].

$$\text{Gl-P} + \text{Gl,6-diP} \rightleftharpoons \text{Gl,6-diP} + \text{G6-P}$$

When the substrate is Gl-P, the phosphate group of the 1-position of Gl,6-diP is transferred to the 6-position of Gl-P. The coenzyme is converted to the product, and the substrate, to the coenzyme[2].

Phosphoglucomutase is widely distributed in living cells and plays an important role on a carbohydrate metabolism as a link of glycolysis and a polysaccharide metabolism. The enzyme was first crystallized from rabbit muscle by Najjar[3].

Reaction mechanism of this enzyme proposed by Najjar et al. involves two steps[4,5],

$$\text{E-P} + \text{Gl-P} \rightleftharpoons \text{E} + \text{Gl,6-diP}$$

$$\text{E} + \text{Gl,6-diP} \rightleftharpoons \text{E-P} + \text{G6-P}$$

where E-P and E represent the phosphorylated and dephosphorylated forms of the enzyme, respectively. This mechanism was based on the formation of G6-P from dephos-

pho-enzyme and Gl,6-diP or of Gl,6-diP from phospho-enzyme and Gl-P[4,5], and was supported by the demonstration of incorporation, from G6-^{32}P in the presence of Gl,6-diP, of ^{32}P into the enzyme[6,7]. This pathway fits "ping-pong" mechanism proposed by Cleland[8]. It was reported by kinetic experiments that the reaction of phosphoglucomutase from rabbit muscle[9], *Escherichia coli*[10,11], shark muscle[12] and flounder muscle[12] proceeded *via* a "ping-pong" pathway.

However, it was observed by the kinetic experiment that the reaction of phosphoglucomutase partially purified from yeast[13] proceeded *via* a "sequential" pathway of Cleland's definition[8].

$$E + \text{Gl-P} + \text{Gl,6-diP} \rightleftharpoons E\begin{matrix}\diagup \text{Gl-P} \\ \diagdown \text{Gl,6-diP}\end{matrix} \rightleftharpoons$$

$$E\begin{matrix}\diagup \text{Gl,6-diP} \\ \diagdown \text{G6-P}\end{matrix} \rightleftharpoons E + \text{Gl,6-diP} + \text{G6-P}$$

It was also reported that the reaction of phosphoglucomutases from *Bacillus cereus* and *Micrococcus lysodeikticus*[11] proceeded *via* a "sequential".

The reaction sequences of phosphoglucomutases from various origins are, therefore, considered not to be identical. The catalytic reaction mechanism of the enzyme should reflect its protein structure. It may be useful

for an understanding of the relationship between the enzyme reaction mechanism and its protein structure that the reaction sequence and the protein structure of phosphoglucomutase purified from yeast are studied in the contrast to the wellknown rabbit muscle enzyme.

Chapter I Purification of Yeast Phosphoglucomutase

Phosphoglucomutase was partially purified from yeast by Najjar[14]. However, the homogeneous preparation of this enzyme had not been obtained, since the enzyme was unstable. It was found that the yeast enzyme activity was stable in citrate buffer (pH 5.3) for 3 days and in Tris buffer (pH 7.5) containing dithiothreitol for one day. Yeast phosphoglucomutase was crystallized after the purification procedures under these conditions.

MATERIALS AND METHODS

Materials Gl-P, G6-P and Gl,6-diP were purchased from Boehringer Mannheim.

Assay of the enzyme activity The enzyme assay was performed under the condition of 1.6 μmoles of Gl-P, 0.01 μ mole of Gl,6-diP and 5.0 μmoles of Tris buffer (pH 7.5), in a final volume of 0.4 ml. After incubation at 30°, the reaction was stopped by the addition of 4.6 ml of 0.56 M H_2SO_4. After heating at 100° for 3 minutes, acid labile phosphate was determined by the method of Fiske et al.[15]. The unit of the activity was expressed as μmole of G6-P produced per minute, and the specific activity was expressed as unit per mg of the protein. The protein

concentration was estimated from the absorbance at 280 nm by assuming the value of $E^{1\%}_{1cm}$ to be 10.

RESULTS

Buffer A, 5.0 mM citrate (pH 5.3), was used throughout purification procedures except for DEAE-Sephadex chromatography. Buffer B, 20 mM Tris buffer (pH 7.5) containing 1.0 mM dithiothreitol and 1.0 mM citrate, was used in DEAE-Sephadex chromatography. Unless otherwise specified, all procedures were undertaken at 4°.

On a typical run, 600g of air dried baker's yeast were mixed with about 500 ml of Buffer A and 1.2 Kg of aluminium oxide and was disrupted by grinding mechanically for about one hour. The viscous suspension was diluted with 1.2 liters of Buffer A, and the extract was obtained by centrifugation. The precipitate was reextracted with 1.2 liters of Buffer A. To the combined extracts (2.1 liters), 1.4 liters of the hot saturated ammonium sulfate solution (90°) were added gradually to bring to 0.4 saturation, and this mixture was kept at 60° for 5 minutes. After cooling on an ice bath, the suspension was filtrated, and to 3.4 liters of filtrate were added 1.04 Kg of solid ammonium sulfate. The precipitate obtained by centrifugation was diluted tc 2.8 liters with

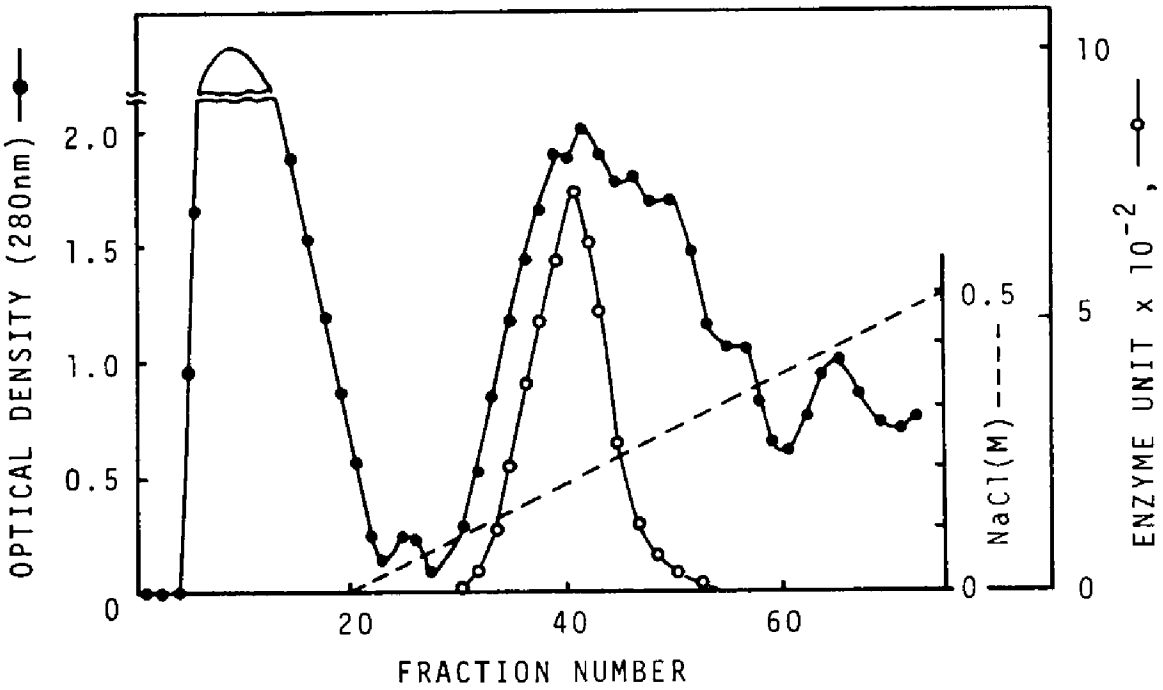

Figure 1 CM-Sephadex Chromatography

The protein was eluted with a linear gradient of NaCl which was ranged from 0 to 0.5 M. Fractions (18 ml) were collected and assayed for protein and activity.

Buffer A to bring the protein concentration to 10 mg per ml, and the solution was fractionated by solid ammonium sulfate (0.55-0.75 saturation). The precipitate was dissolved in a small volume of Buffer A, and salt was removed from the solution by passage through a Sephadex G-50 column (3.5 x 100 cm) equilibrated with Buffer A. The enzyme solution was applied to a CM-Sephadex column

(3 x 42 cm) previously equilibrated with Buffer A. After washing the column with Buffer A, the enzyme was eluted with 800 ml of linear gradient of NaCl in Buffer A. Figure 1 represents a typical elution profile showing protein concentration and enzyme activity. Fractions having a high specific activity (Fractions, No.34-43) were collected, and the protein was precipitated by the addition of solid ammonium sulfate (0.75 saturation). The precipitate was diluted with Buffer A to bring the protein concentration to 12 mg/ml, and the solution was fractionated with solid ammonium sulfate (0.5-0.6 saturation). The precipitate was dissolved in a small volume of Buffer A, and the solution was passed through a Sephadex G-50 column (1.7 x 45 cm) equilibrated with Buffer B. The enzyme solution was applied to DEAE-Sephadex column (1.5 x 20 cm) previously equilibrated with Buffer B. The enzyme was eluted with 250 ml of Buffer B, and the phosphoglucomutase activity was observed at the second peak of fractions as shown in Figure 2. The active fractions (Fractions, No.15-33) were collected, and the protein was precipitated by the addition of solid ammonium sulfate (0.75 saturation). The precipitate was dissolved in Buffer A containing 1.0 mM dithiothreitol to bring the protein concentration to about

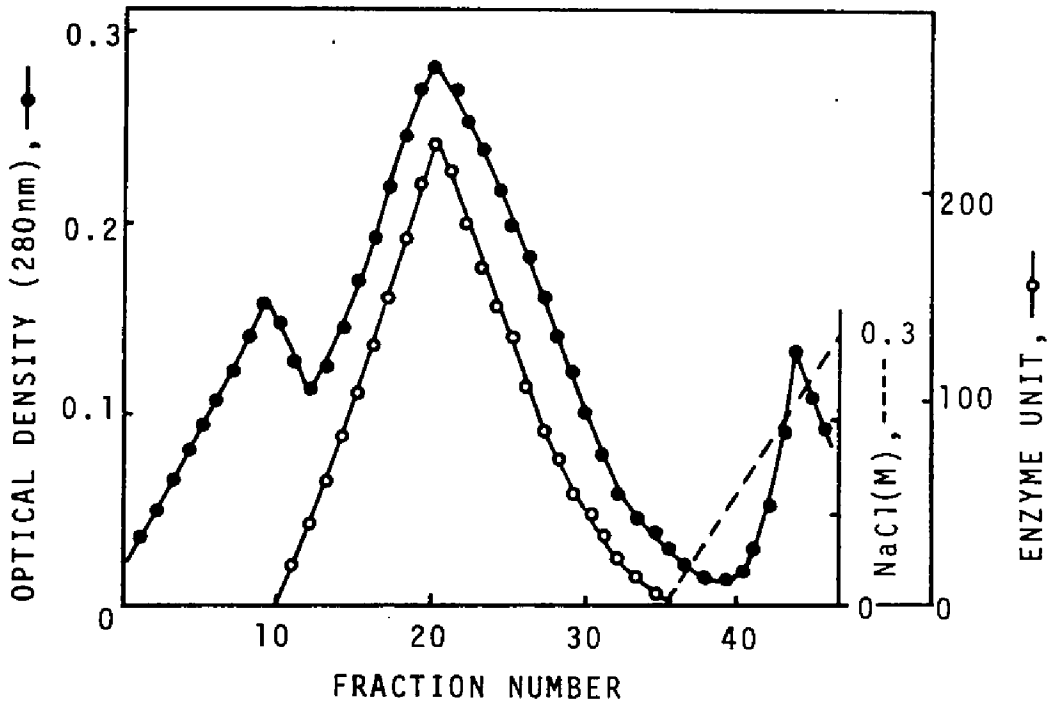

Figure 2 DEAE-Sephadex Chromatography

Fractions (6.0 ml) were collected and assayed for protein and activity.

10 mg/ml, and to the clear enzyme solution the saturated ammonium sulfate solution was added gradually. The material showing a faint turbidity was allowed to stand for a few days. The further addition of the saturated ammonium sulfate solution gave the material deeper turbidity and silky sheen. Within a few weeks crystallization was completed. The crystalline form of the

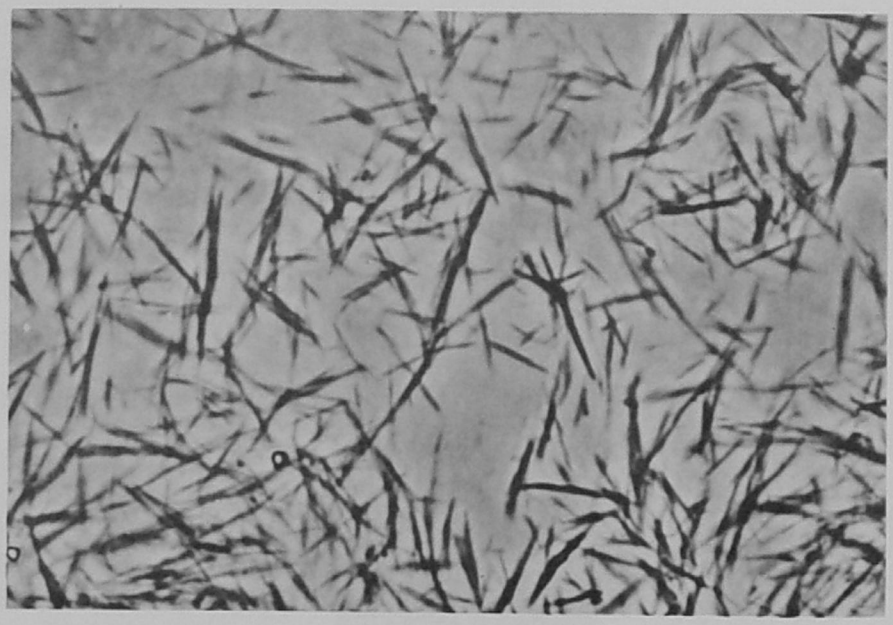

Figure 3 Crystals of Yeast Phosphoglucomutase

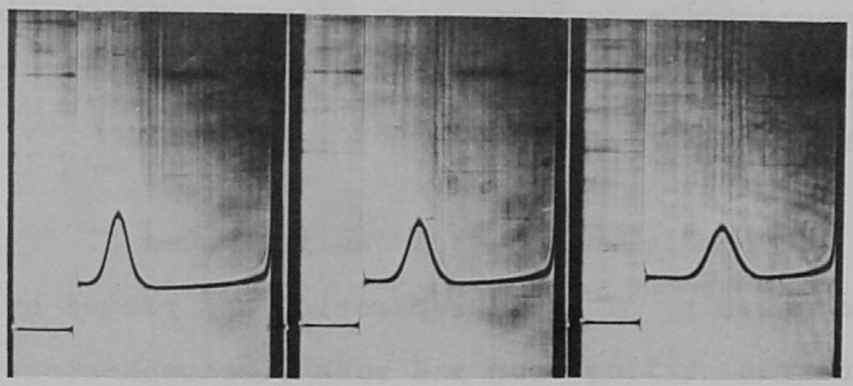

Figure 4 Ultracentrifugal Patterns of Yeast Phosphoglucomutase

The experiments of ultracentrifugation were performed at pH 5.3 (5 mM citrate) and 10°. Concentration of the enzyme was 10 mg/ml. Ionic strength was adjusted by NaCl to 0.1.

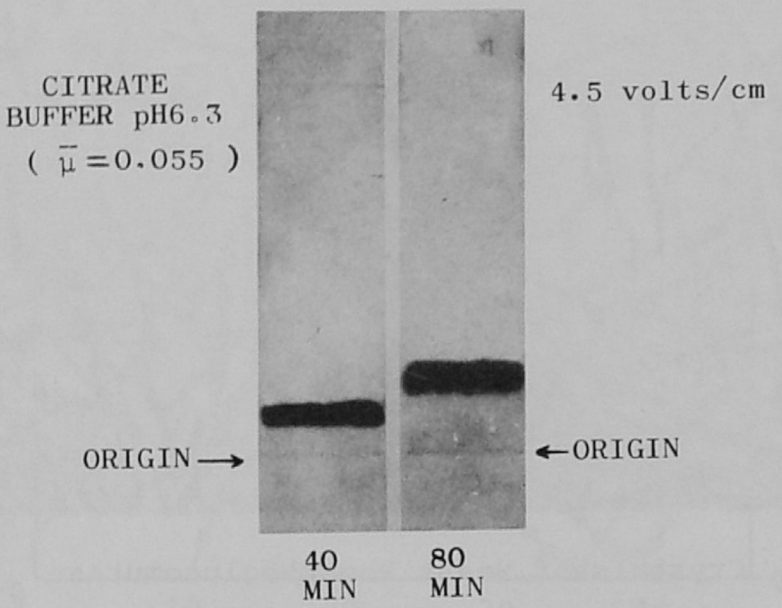

Figure 5 . Zone Electrophoresis

Electrophoresis was performed on 10 cm strips of cellulose-acetate membrane. After electrophoresis the protein was stained with Amidoblack 10B.

enzyme was needle-shaped as shown in Figure 3.

Homogeneity of this preparation was proved by analytical ultracentrifugation and zone electrophoresis as shown in Figure 4 and 5.

The specific activity of the crystalline preparation was 193 under the standard condition. The specific activity of yeast phosphoglucomutase purified by Najjar[14] was 59. The activity of the yeast enzyme was stimu-

TABLE I PURIFICATION OF YEAST PHOSPHOGLUCOMUTASE

Procedures	Protein* (mg)	Enzyme** units	Specific activity	Yield (%)
Crude extracts	—	25,000	—	100
Heat treatment	28,000	19,000	0.70	75
Ammonium sulfate fractionation	8,300	11,000	1.33	44
CM-Sephadex chromatography	400	7,700	19.1	31
Ammonium sulfate fractionation	66	5,500	84.0	22
DEAE-Sephadex chromatography	27	5,000	185	20
Crystallization	20	3,850	193	15

*Estimated from absorbance at 280 nm by assuming the value of $E^{1\%}_{1cm}$ to be 10.

**μmoles of G6-P produced per minute.

lated about 1.3 fold by the addition of EDTA as shown in Chapter V. Thus the specific activity of the crystalline preparation was 250 under the optimal condition. This value was near that of rabbit muscle enzyme under the optimal condition[16,17].

Chapter II Reaction Mechanism of Yeast Phosphoglucomutase

It was reported by Najjar et al. that the reaction of yeast phosphoglucomutase proceeded *via* a "ping-pong" pathway[14]. This was based on the demonstration of the formations of G6-P from dephospho-enzyme and Gl,6-diP or of Gl,6-diP from phospho-enzyme and G6-P. However, the validity of the mechanism for the yeast enzyme cannot be considered to be unequivocally confirmed because of the inhomogeneity of the enzyme preparation or the limitation of the experimental method.

Kinetic studies can distinguish between "sequential" and "ping-pong" pathway, since in "sequential" pathway each line of double reciprocal plots meets at a point when substrate is varied at several fixed level of coenzyme, and *vice versa*, while in "ping-pong" pathway is parallel. In the kinetic experiment with use of phosphoglucomutase partially purified from yeast, each line of the double reciprocal plots met at a point[13].

In this chapter, the results of further studies on the reaction mechanism of purified yeast phosphoglucomutase are described.

MATERIALS AND METHODS

Materials G6-P dehydrogenase and NADP were purchased from Boehringer Mannheim and Sigma, respectively. Gl-P was purified by the method of Ray et al.[9]. The purified Gl-P did not have any contamination of Gl,6-diP, since phosphoglucomutase activity was not observed without the addition of Gl,6-diP.

Crystalline yeast phosphoglucomutase was prepared as described in Chapter I.

Assay of the enzyme activity The reaction mixture contained 0.016-3.2 μmoles of Gl-P, 0.72-20 nmoles of Gl,6-diP, 0.4 μmole of NADP, 20 μmoles of Tris buffer (pH 7.5), 20 μmoles of $MgCl_2$, 0.01 μmole of EDTA and 18 μg of G6-P dehydrogenase in a final volume of 2.0 ml. The preparation of G6-P dehydrogenase was free from phosphoglucomutase, since the absorbance at 340 nm was not increased without the addition of phosphoglucomutase under this condition. After preincubation in a cuvette of 1cm light path at 25°, the reaction was started by the addition of the enzyme dissolved in 1 mM citrate buffer (pH 5.3) containing 50 μM dithiothreitol. The increase of absorbancy at 340 nm was recorded with Hitachi Double-Beam Spectrophotometer Model-124 equipped with a temperature controlled cell-holder.

Error in the coupled reaction When the reaction velocity of phosphoglucomutase is determined by the coupled reaction, an amount of G6-P dehydrogenase is the limiting factor to the experimental error. When v_1 and v_2 represent the velocities of phosphoglucomutase and G6-P dehydrogenase reaction, respectively,

$$\text{Gl-P} \xrightarrow[v_1]{} \text{G6-P} \xrightarrow[v_2]{} \text{6-phosphogluconate}$$

v_1 equals to v_2 within the range of linear increase of absorbance at 340 nm (steady-state). Therefore, the following equation is obtained.

$$\frac{[S]V_{max,1}}{[S] + Km_1} = \frac{f[S]V_{max,2}}{f[S] + Km_2}$$

$$f = \frac{Km_2}{V_{max,2}/V_{max,1}([S] + Km_1) - [S]}$$

Where [S] and f[S] represent the concentrations of Gl-P and G6-P at steady-state, and $V_{max,1}$ and $V_{max,2}$, the maximum velocity of phosphoglucomutase and G6-P dehydrogenase, and Km_1 and Km_2, the Km value of phosphoglucomutase to Gl-P and G6-P dehydrogenase to G6-P, respectively. The error of the substrate concentration is expressed by f/(f+1). Thus the experimental condition must be designed to minimize the f value. The above equation

is simplified, since $V_{max,2}$ is much larger than $V_{max,1}$ as described below.

$$f = \frac{Km_2}{Km_1 + [S]} \cdot \frac{V_{max,1}}{V_{max,2}}$$

With use of this equation and the results in Figure 6, the maximum error of the substrate concentration can be calculated. Under the conditions of the enzyme assay, Km value of G6-P dehydrogenase to G6-P was determined (Km_2 = 36 μM). $V_{max,2}$ was determined to be 5.6 optical density change per minute. $V_{max,1}$ was calculated to be 0.04 optical density change per minute and the minimum value of apparent Km_1, 8.0 μM (see Fig.6).

$$f = \frac{36}{8.0 + [S]} \cdot \frac{0.04}{5.6}$$

When the initial concentration of Gl-P is diluted infinitely, the value of [S] must be nearly equal to zero. [S] could be regarded as the initial concentration of Gl-P, since the maximum value of f (in the case of [S]≈0) was small enough compared with 1 (f=0.032). Thus the f value at the minimum concentration of Gl-P (8.0 μM) was 0.016. The maximum error of Gl-P concentration was about 1.6 %. The amount of G6-P dehydrogenase used in the assay was enough to neglect the error in the

coupled reaction method.

RESULTS

The activities of yeast phosphoglucomutase were measured in 8.0 μM – 0.1 mM Gl-P and 0.36 μM – 10 μM Gl,6-diP. Figure 6a shows Lineweaver-Burk plots of 1/v *versus* 1/[Gl,6-diP] at four concentrations of Gl-P, and Figure 6b, 1/v *versus* 1/[Gl-P] at five concentrations of Gl,6-diP.

In both figures the lines met at a point on the third quadrant. This result indicates that the reaction of yeast phosphoglucomutase fits "sequential" mechanism.

The deviation from straight lines at higher substrate concentrations as shown in Figure 6b suggests that the substrate inhibition occurs. Figure 6a shows that the coenzyme inhibition does not occur within concentrations of Gl,6-diP used.

When the concentration of substrate was changed from 0.04 – 1.6 mM, Lineweaver-Burk plots of 1/v *versus* 1/[Gl,6-diP] at the various concentrations of Gl-P gave straight lines which met at a point on the ordinate. This result indicates that the substrate inhibition in the yeast phosphoglucomutase reaction is due to the binding

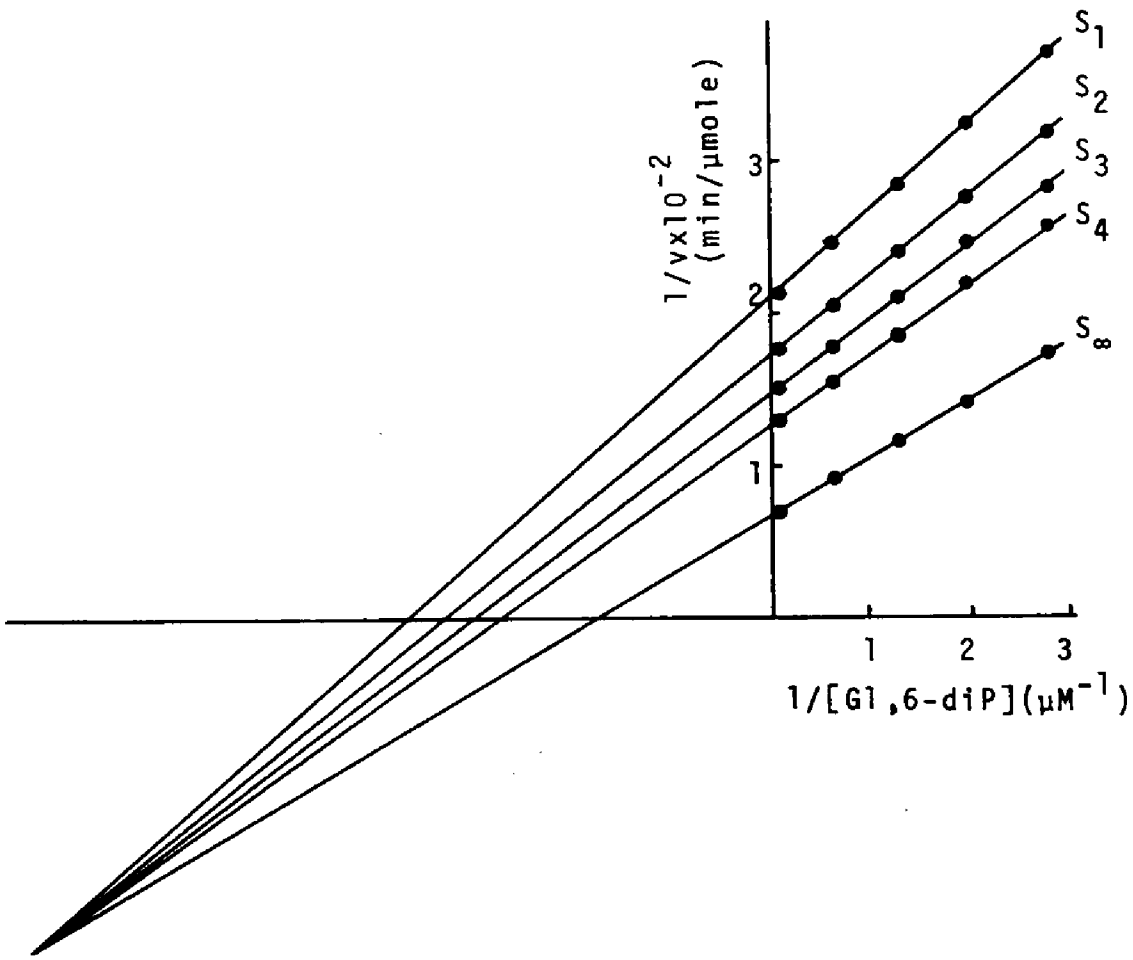

Figure 6a Lineweaver-Burk plot of 1/v *versus* 1/[Gl,6-diP]

Initial velocity,v, is represented as μmoles of NADPH produced per minute. The value of 1/v at S_∞ were obtained from the intercept of lines on the ordinate in Fig.6b.

The concentrations of Gl-P : S_1, 8.0 μM ; S_2, 10 μM ; S_3, 14 μM ; S_4, 20 μM.

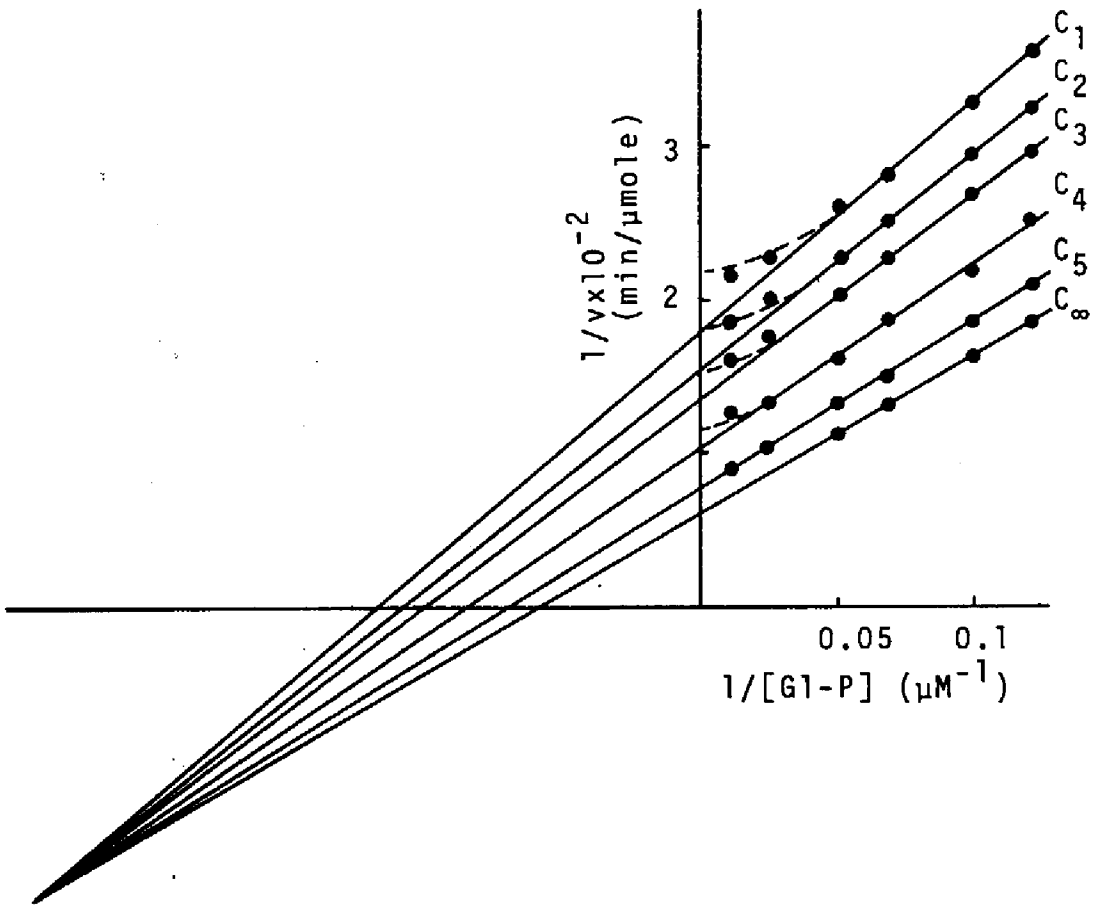

Figure 6b Lineweaver-Burk plot of 1/v *versus* 1/[G1-P]

The value of 1/v at C_∞ were obtained from the intercept of lines on the ordinate in Fig.6a.

The concentrations of G1,6-diP : C_1, 0.36 μM ; C_2, 0.5 μM ; C_3, 0.7 μM ; C_4, 1.4 μM ; C_5, 10 μM.

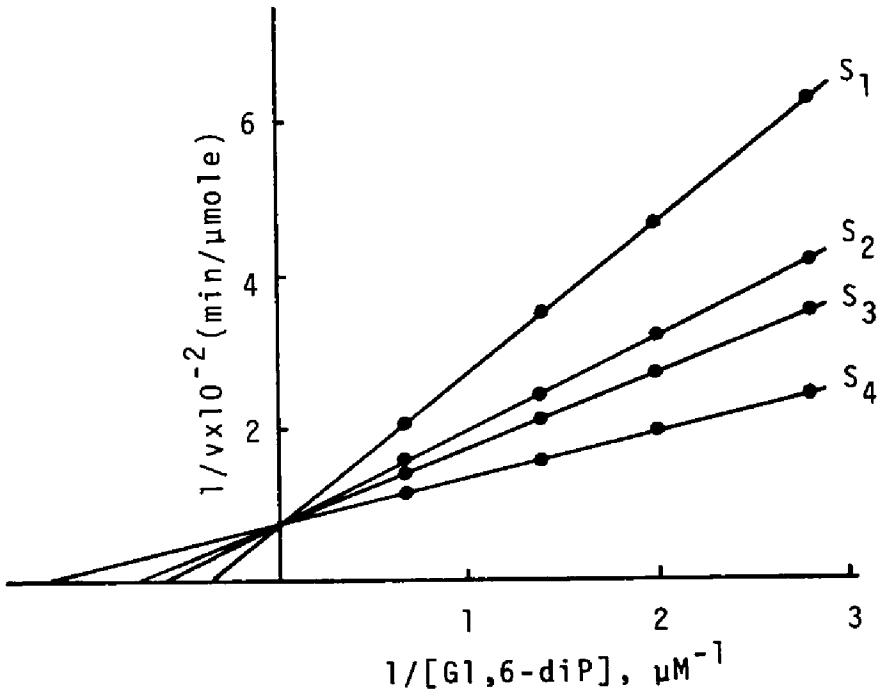

Figure 7 Lineweaver-Burk Plot in the Yeast Phosphoglucomutase Reaction under Occurrence of Substrate Inhibition

The concentrations of Gl-P : S_1, 1.6 mM ; S_2, 1.0 mM ; S_3, 0.6 mM ; S_4, 0.04 mM.

of substrate to coenzyme binding site (Fig.7).

"Sequential" mechanism is divided into "random sequential" and "ordered sequential" mechanism[8]. When Gl-P combines only with enzyme·Gl,6-diP complex in "ordered sequential" mechanism,

$$E \underset{k_{-1}}{\overset{k_1[C]}{\rightleftharpoons}} EC \underset{k_{-2}}{\overset{k_2[S]}{\rightleftharpoons}} ECS \xrightarrow{k_3} E + \text{product}$$

the rate equation is given as follows.

$$v = \frac{[S][C]V_{max}}{[S][C] + \frac{k_{-2}+k_3}{k_2}[C] + \frac{k_3}{k_1}[S] + \frac{k_{-1}(k_{-2}+k_3)}{k_1 k_2}}$$

Where E, S, C and k's represent the enzyme, Gl-P, Gl,6-diP and the rate constants on each steps of reactions, respectively. Each line in Lineweaver-Burk plots of 1/v *versus* 1/[S] at various concentrations of C meets at a point*. The point exists on the second quadrant, on the abscissa and on the third quadrant in cases of $k_{-1} > k_3$, $k_{-1} = k_3$ and $k_{-1} < k_3$, respectively. If the reaction mechanism of yeast phosphoglucomutase is "ordered sequential", the condition of $k_3 > k_{-1}$ is required in order to explain the results of Figure 6.

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*This treatment is also applicable to the case that Gl,6-diP combine only with the enzyme·Gl-P complex.

In "random sequential" mechanism*, the enzyme species are as follows :

$$E + S \rightleftharpoons ES \; ; \; Ks$$

$$E + C \rightleftharpoons EC \; ; \; Kc$$

$$EC + S \rightleftharpoons ECS \; ; \; \alpha Ks$$

$$ES + C \rightleftharpoons ECS \; ; \; \alpha Kc$$

$$ECS \xrightarrow{k} E + \text{product}$$

Where k is the rate constant of breakdown of ECS complex, and Ks, Kc, αKs and αKc are the dissociation constants of the respective steps**. In this mechanism, S-binding affects the affinity of C to the enzyme, and *vice versa* (e.g. in the case of $\alpha > 1$, the binding of S decreases the affinity of C to the enzyme, and *vice versa*). The rate equation for "random sequential" mechanism is represented below.

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* In "random sequential" mechanism, the rate equation derived from steady-state method contains square terms, and reciprocal plots are not straight lines. However, Lineweaver-Burk plot in yeast phosphoglucomutase reaction was linear unless the substrate inhibition occurred. Thus "random sequential" mechanism means "rapid equilibrium random sequential" mechanism.

**When the dissociation constant of fourth of above equations is βKc, β is expressed as [ES][C]/[ECS]Kc (= [E][S][C]/[ECS]KcKs). On the other hand, α is equal to [EC][S]/[ECS]Ks (=[E][C][S]/[ECS]KsKc). Therefore α=β.

$$v = \frac{[S][C]V_{max}}{\alpha KsKc\{ \frac{[S]}{Ks} (1 + \frac{[C]}{\alpha Kc}) + \frac{[C]}{Kc} + 1 \}}$$

Lineweaver-Burk plots of 1/v *versus* 1/[S] at the various concentrations of C give straight lines and *vice versa*. The straight lines meet at a point on the second quadrant, on the abscissa and on the third quadrant under the conditions of $\alpha < 1$, $\alpha = 1$ and $\alpha > 1$, respectively. The value of -1/Ks or -1/Kc can be calculated from the meeting point of each line. The values of -1/αKs and -1/αKc can be obtained from the intercept on the abscissa of the lines at C_∞ and S_∞, respectively.

From Figure 6a and b, Ks = 4.0 μM, Kc = 0.14 μM and α = 4.1 were obtained. If the yeast phosphoglucomutase reaction obeys "random sequential" mechanism, the binding of Gl-P should decrease the affinity of Gl,6-diP to the enzyme, and *vice versa*.

DISCUSSION

If the yeast phosphoglucomutase reaction occurs with "ordered sequential" mechanism, it is required that the rate constant of the breakdown of ECS complex (k_3) is larger than the rate constant of the dissociation of EC

or ES (k_{-1}), which seems unlikely. Although "ordered sequential" mechanism can not be necessarily excluded from the yeast phosphoglucomutase reaction pathway, it is unlikely that the yeast phosphoglucomutase reaction proceeds *via* "ordered sequential" mechanism. Indeed, as described in Chapter IV, the results of fluorimetric titration suggested that the reaction of yeast phosphoglucomutase proceeded *via* "random sequential".

The constant (α) in "random sequential" mechanism posesses the important sense in the interpretation of the kinetics of phosphoglucomutase. When the value of α is large enough (e.g., $\alpha > 100$), Lineweaver-Burk plot gives a parallel pattern apparently. Therefore, "ping-pong" mechanism is not necessarily justified by the parallel pattern in Lineweaver-Burk plot.

Najjar regarded the purified enzyme as the phosphorylated enzyme, and demonstrated the formation of Gl,6-diP from phospho-enzyme and Gl-P (or G6-P) as shown in the equation in Introduction[3,4]. Dephospho-enzyme was prepared by dialysis of phospho-enzyme against Gl-P[3,4]. However, in the case of α value being large enough, Gl-P strongly decrease the affinity of Gl,6-diP to the enzyme, and *vice versa*. If the purified phosphoglucomutase is not phospho-enzyme but the firmly bound complex of the

enzyme and Gl,6-diP, Gl,6-diP will be released from the enzyme·Gl,6-diP complex by the addition of Gl-P.

$$E\cdot Gl,6\text{-}diP + Gl\text{-}P \rightleftharpoons E\cdot G6\text{-}P + Gl,6\text{-}diP$$

By the addition of Gl,6-diP, G6-P will be released from the enzyme·G6-P complex prepared by dialysis against Gl-P (or G6-P).

$$E\cdot G6\text{-}P + Gl,6\text{-}diP \rightleftharpoons E\cdot Gl,6\text{-}diP + G6\text{-}P$$

Although "ping-pong" mechanism supported by the demonstration of incorporation, from G6-^{32}P in the presence of Gl,6-diP, of ^{32}P into the enzyme[6,7], it does not always follow that the incorporated ^{32}P into the enzyme is not in the form of E·Gl,6-di^{32}P, but E-^{32}P.

It was suggested by the experiments of incorporation of D-2,3-diphosphoglycerate-^{32}P, the coenzyme of phosphoglyceromutase, into rabbit muscle phosphoglyceromutase that the bound radioactivity was not in the form of the enzyme-phosphate, but in the form of the enzyme·coenzyme[18]. In fact, it was found that yeast phosphoglyceromutase showed the enzyme·coenzyme·substrate ternary complex as an active intermediate in kinetic experiments[19].

Although a "ping-pong" pathway of rabbit muscle phosphoglucomutase are not always denied by these suggestions,

conclusive studies for excluding this "random sequential" mechanism have not been carried out in the muscle enzyme reaction.

Chapter III Coenzymatic Activity of Fructose 1,6-diphosphate

As shown in Introduction, phosphoglucomutase requires Gl,6-diP as a coenzyme. It was found that the reaction of yeast phosphoglucomutase proceeded in the absence of Gl,6-diP with the addition of Fl,6-diP. It is considered to be important for understanding of the regulation of carbohydrate metabolism that the mechanism of coenzymatic activity of Fl,6-diP are clarified. In this chapter, the formation of Gl,6-diP from Gl-P and Fl,6-diP in the yeast phosphoglucomutase reaction is described.

MATERIALS AND METHODS

Reagents Gl-P, G6-P,Gl,6-diP, Fl,6-diP, G6-P dehydrogenase and NADP were purchased from Boehringer Mannheim. Gl-P was purified chromatographically by the method of Ray et al.[9]. Fl,6-diP was used after acid-hydrolysis (1M HCl, 100°, 3 minutes) to exclude the effect of Gl,6-diP which might contaminate the Fl,6-diP preparation.

Crystalline yeast phosphoglucomutase was prepared as described in Chapter I.

Assay of the enzyme activity Unless otherwise specified, the reaction mixture contained 0.2 μmole of Gl-P,

5 μmoles of Tris buffer (pH 7.5) and various amounts of Fl,6-diP or Gl,6-diP in a final volume of 0.5 ml. The reaction at 25° was started by the addition of the enzyme. After the reaction was stopped by the addition of 3.5 ml of 0.6M H_2SO_4, the solution was heated at 100° for 3 minutes. Acid labile phosphate was determined by the method of Chen et al.[20].

RESULTS

Coenzymatic activity of Fl,6-diP

Coenzymatic activities of acid-hydrolyzed Gl,6-diP and Fl,6-diP were examined. It was determined from the amounts of inorganic phosphate that Gl,6-diP was completely hydrolyzed by heating at 100° in 1M HCl for 3 minutes, and that about 30 percent of Fl,6-diP was hydrolyzed under the same condition. Table II shows that the coenzymatic activity of Gl,6-diP was lost by the acid treatment, and Fl,6-diP, was not. This indicates that the coenzymatic activity of Fl,6-diP is not due to Gl,6-diP which may be present in the Fl,6-diP preparation.

The conversion of Gl-P to G6-P was confirmed in the Fl,6-diP dependent reaction of the enzyme. Gl-P was determined from the amount of acid-labile phosphate, and

TABLE II COENZYMATIC ACTIVITY OF ACID-HYDROLYZED Fl,6-DIP AND Gl,6-DIP

For the measurements of the activity, 0.45 μg of the enzyme was used. Acid-hydrolyzed coenzymes were obtained as follows. Fl,6-diP was heated at 100° in 1M HCl for 3 minute and neutralized with 1M NaOH.

Coenzyme	Treatment	Activity (nmole/min)
Gl,6-diP (1.0 μM)	none	54.2
	acid hydrolysis	0
Fl,6-diP (60 μM)	none	21.6
	acid hydrolysis	19.5

G6-P, from the amount of NADPH produced by G6-P dehydrogenase. Figure 8 shows that the increase of G6-P agreed well with the decrease of Gl-P. This result suggests that the main reaction in the Fl,6-diP dependent reaction of the enzyme is a conversion of Gl-P to G6-P.

Formation of Gl,6-diP

The time course of the Fl,6-diP dependent reaction was linear after a time lag. This suggested that the

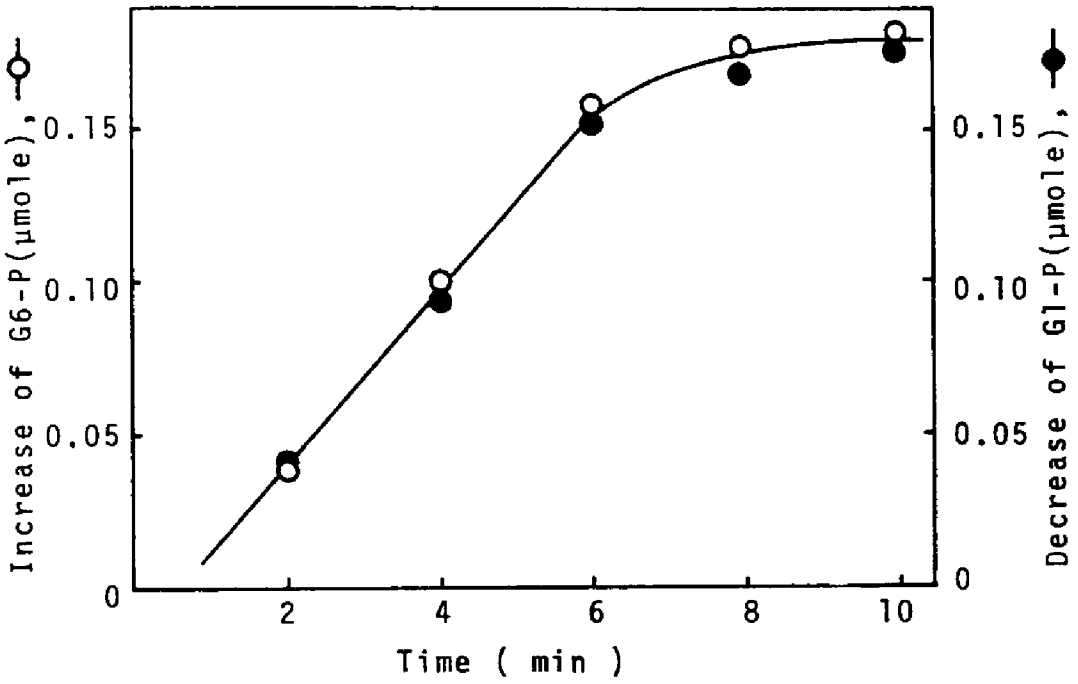

Figure 8 Fl,6-diP Dependent Reaction of Yeast Phosphoglucomutase

Reactions were carried out in 0.2 μmole of Gl-P, 0.4 μmole of Fl,6-diP, 20 μmole of Tris buffer (pH 7.5) and 0.4 μg of the enzyme at 25° in a final volume of 2.0 ml. In the determination of G6-P, 20 μg of G6-P dehydrogenase and 0.8 μmole of NADP were added. The decrease of Gl-P was determined by the measurement of acid-labile phosphate, the increase of G6-P, by measuring the absorption of NADPH at 340 nm.

Symbols : ● , decrease of Gl-P ; o , increase of G6-P.

two types of the reaction participated in the Fl,6-diP dependent reaction. In the second reaction, Gl-P was

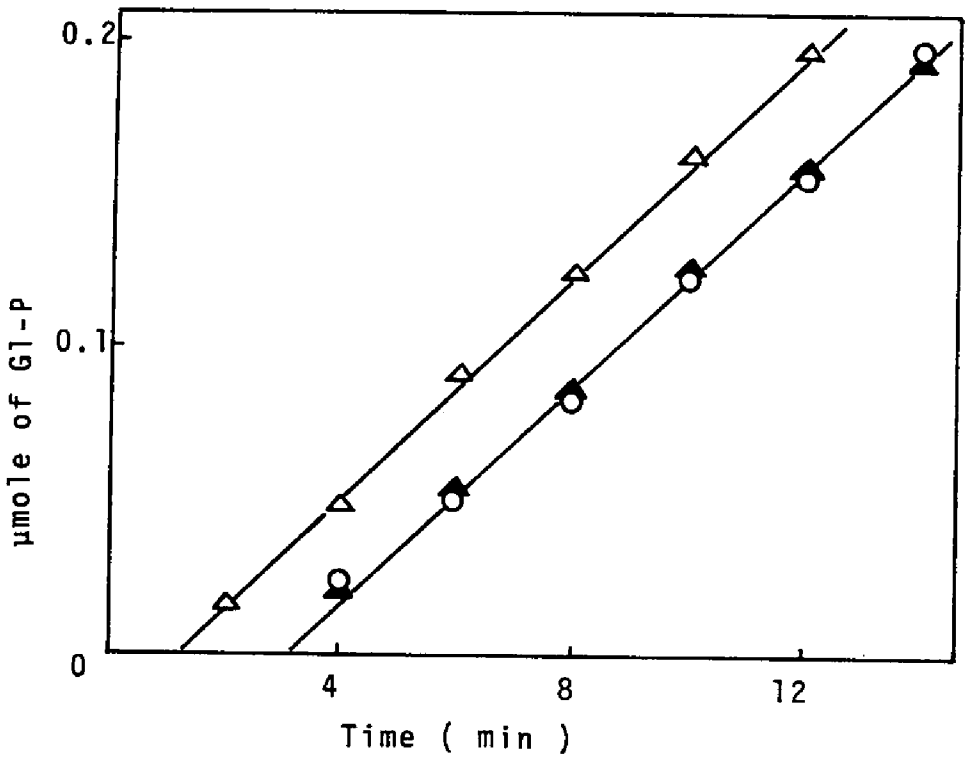

Figure 9 The Time Lag in the Fl,6-diP Dependent Reaction

The reaction were carried out as described in the text. The concentration of Fl,6-diP in the reaction mixture was 2.5 µM. The enzyme was preincubated with Fl,6-diP and Gl,6-diP for 30 minutes at 25°.

Addition to the preincubation mixture :
O ,none ; ▲ , 2.5 µM Fl,6-diP ; Δ , 2.5 µM Fl,6-diP and 0.4 mM Gl-P.

converted to G6-P as shown in Figure 8. To clarify the first step of the reaction, the enzyme was preincubated with Fl,6-diP or with Gl-P and Fl,6-diP. The time lag was largely diminished only in the case that the enzyme was preincubated with Gl-P and Fl,6-diP as shown in Figure 9. This suggests that in the first step of the reaction the enzyme-phosphate complex is not formed, but the direct phosphate transfer of Fl,6-diP to Gl-P occurs as shown in the following reaction.

$$\text{Gl-P} + \text{Fl,6-diP} \rightleftharpoons \text{Gl,6-diP} + \text{F6-P (or Fl-P)}$$

This reaction is reasonably interpreted by "sequential" pathway in the yeast phosphoglucomutase reaction.

The formation of Gl,6-diP with the reaction was confirmed as follows. Fl,6-diP is destroyed by alkali-treatment, and Gl,6-diP is relatively stable under the treatment. Fl,6-diP was incubated with the enzyme or with Gl-P and the enzyme. After an alkali-treatment of the solutions, the coenzymatic activities were measured. Table III shows that the coenzymatic activity is not lost in the case that Fl,6-diP is incubated with Gl-P and the enzyme. These results indicate that yeast phosphoglucomutase is able to catalyze the phosphate-transfer of Fl,6-diP to Gl-P.

TABLE III COENZYMATIC ACTIVITY OF ALKALI-HYDROLYZED Fl,6-DIP AND Gl,6-DIP

The coenzymatic activity of Fl,6-diP and Gl,6-diP were measured with use of 0.54 µg of the enzyme after the alkali-hydrolysis of the coenzymes which were previously incubated at 25° with 0.50 µg of the enzyme and 1.2 µmoles of Gl-P in the volume of 3.0 ml. Alkali-hydrolyzed coenzymes were prepared as follows. The coenzyme solutions were heated at 90° in 1M NaOH for 1 hour and were neutralized with Amberlite IR-120 (H^+-form).

Coenzyme	Preincubation Addition	Time(min)	Treatment	Activity (nmole/min)
Fl,6-diP (10 µM)	none	—	none	19.0
	none	—	alkali	0
	enzyme	75	"	0
	enzyme,Gl-P	0	"	0
	enzyme,Gl-P	75	"	12.1
Gl,6-diP (0.2 µM)	none	—	none	43.0
	enzyme,Gl-P	75	alkali	22.0

Kinetic constants

Figure 10 shows that the velocity of Fl,6-diP dependent reaction is increased, and the time lag is

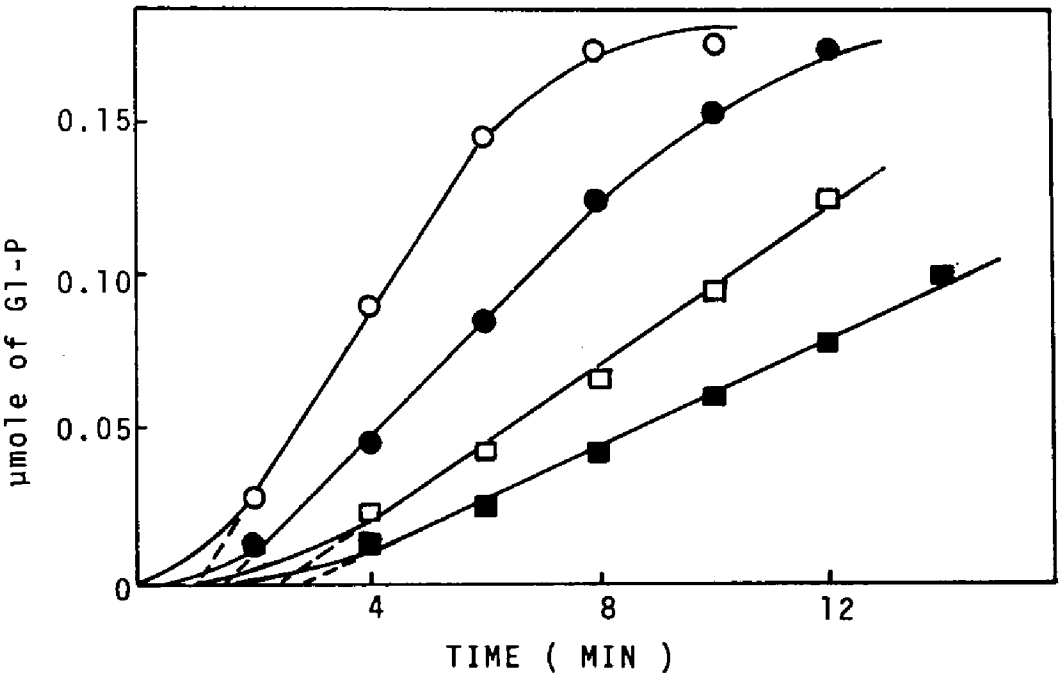

Figure 10 Time Courses of Fl,6-diP Dependent Reaction

The reactions were carried out as described in the text.

Concentrations of Fl,6-diP : ○ , 0.10 mM ; ● , 10 μM ; □ , 5.0 μM ; ■ , 2.5 μM.

decreased with an increment of the concentration of Fl,6-diP added. Lineweaver-Burk plot of $1/v_{app}$ *versus* 1/[Fl,6-diP] was linear as shown in Figure 11. The apparent velocity was obtained from the slope of linear increase in Figure 10.

The v_{app} values should be the velocities of the

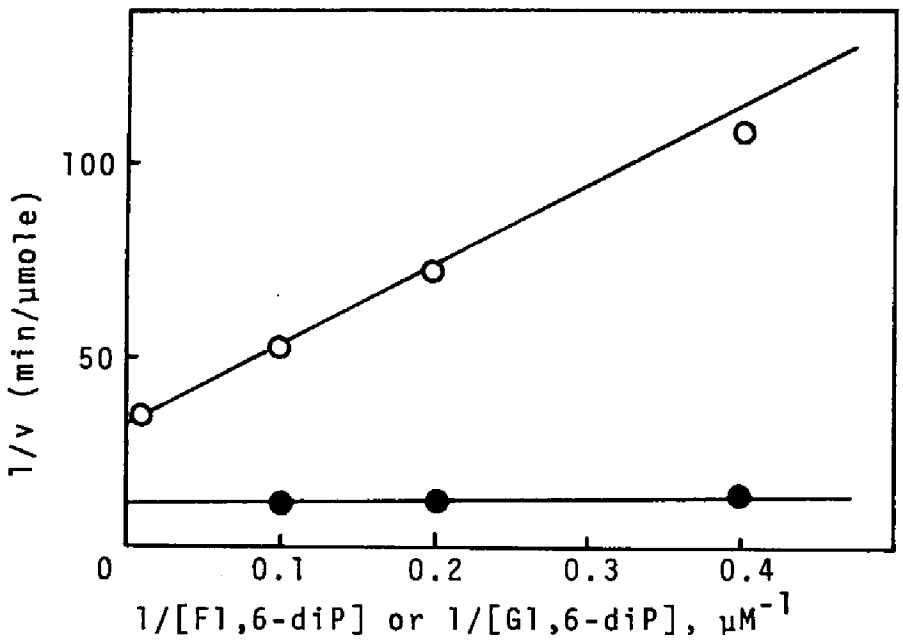

Figure 11 Lineweaver-Burk Plot of Yeast Phosphoglucomutase Reaction

The reactions were carried out as described in the text.

Symbols : ○ , Fl,6-diP ; ● , Gl,6-diP.

reaction in the presence of Gl,6-diP which is formed in the reaction of the formation of Gl,6-diP as concluded from the results of Figure 8 and Table III. Thus the concentration of Gl,6-diP, x, formed within the time lag can be calculated from the following equation.

$$v_{app} = \frac{x \cdot V_{max,g}}{Km_g + x}$$

Where $V_{max,g}$ and Km_g are the maximum velocity and Km value for Gl,6-diP (0.14 μM) in the Gl,6-diP dependent reaction, respectively. Therefore, the velocity in the reaction of the formation of Gl,6-diP, v_f, can be obtained.

$$v_f = \frac{x}{T}$$

Where T represents tha time lag in the Fl,6-diP dependent reaction. The values of T were obtained from the intercepts of the dotted lines on the abscissa in Figure 10, and x, from Figure 11. The values of v_f were calculated from this equation. Figrue 12 shows that the Lineweaver-Burk plot of $1/v_f$ *versus* 1/[Fl,6-diP] is linear. The Km_f value, Km for Fl,6-diP in the reaction of the formation of Gl,6-diP, was 40 μM, and $V_{max,f}$, the maximum velocity in the reaction of the formation of Gl,6-diP, was 0.04 nmole/min. These indicate that Km_f/Km_g is 290 and $V_{max,f}/V_{max,g}$ is 1/530 (see Fig.11).

It was demonstrated that in the Fl,6-diP dependent reaction of the enzyme, the slow reaction of the formation of Gl,6-diP occurred in the first step, and the fast reaction of the original mutase, in the second step.

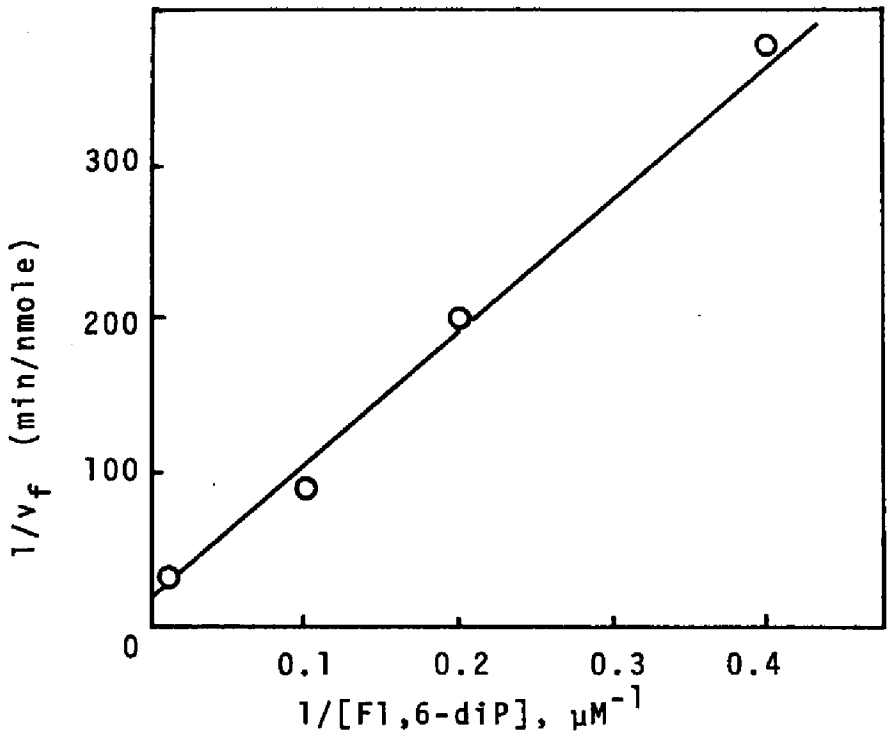

Figure 12 Lineweaver-Burk Plot of $1/v_f$ *versus* 1/[Fl,6-diP]

The values of v_f were calculated from the equation in the text.

The time course of the overall reaction is theoretically shown as follows. The velocity of the formation of Gl,6-diP, dx/dt, is shown as the following equation, since Fl,6-diP should bind to the Gl,6-diP binding site of the enzyme.

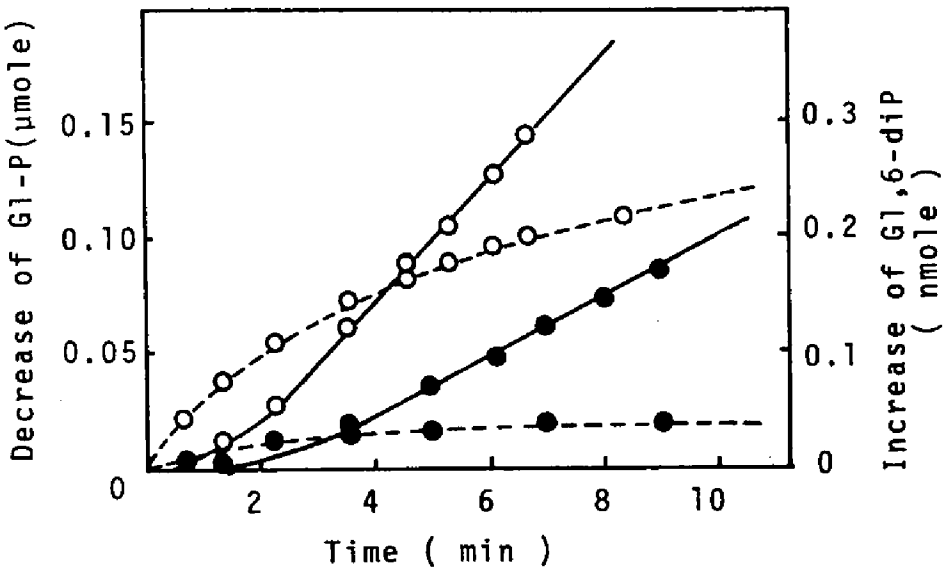

Figure 13 Theoretical Time Courses of Fl,6-diP Dependent Reaction

The solid lines and the dotted lines represent the decrease of Gl-P and the increase of Gl,6-diP, respectively.

The concentration of Fl,6-diP : ○ , 0.1 mM ; ● , 2.5 μM.

$$\frac{dx}{dt} = \frac{([F]_0 - x)V_{max,f}}{([F]_0 - x) + Km_f(1 + \frac{x}{Km_g})}$$

Where $[F]_0$ represents the initial concentration of Fl,6-diP. This equation is changed by integration.

$$\left(1 - \frac{Km_f}{Km_g}\right)\cdot x - Km_f\left(1 + \frac{[F]_0}{Km_g}\right)\cdot \ln\left(1 - \frac{x}{[F]_0}\right) = V_{max,f}\cdot t$$

This equation is simplified as follows, since $[F]_0$ and Km_f are large compared with Km_g.

$$-\frac{x}{[F]_0} - \ln\left(1 - \frac{x}{[F]_0}\right) = \frac{V_{max,f}\cdot Km_g}{[F]_0\cdot Km_f}\cdot t$$

Figure 13 shows that the theoretical time courses calculated from the constants obtained from the experimental results are linear after a time lag, in spite of the fact that the amount of Gl,6-diP increases with a proceeding of time. The time courses in Figure 13 agreed well with Figure 10. This indicates that the Fl,6-diP dependent reaction of yeast phosphoglucomutase is due to the formation of Gl,6-diP.

DISCUSSION

It was reported in the reaction of the rabbit muscle enzyme that 1,3-diphosphoglycerate[21,22] and Fl,6-diP[23,24] are also able to act as a coenzyme. This was interpreted by the formation of the enzyme-phosphate complex, the active intermediate in "ping-pong" pathway, from

dephospho-enzyme and the diphosphate compounds[21-24].
It was shown in this chapter that Gl,6-diP could be produced from Gl-P and Fl,6-diP in the yeast phosphoglucomutase reaction which proceeded *via* "sequential" pathway.

This reaction is important for a sugar metabolism of yeast on the point that the coenzyme of phosphoglucomutase can be produced from Fl,6-diP of the main intermediate of glycolysis.

Chapter IV Fundamental Properties and Chemical Modification of Yeast Phosphoglucomutase

It was shown in Chapter II that the reaction mechanisms of phosphoglucomutases from yeast and rabbit muscle were not identical in spite of the fact that the both enzymes catalyzed the identical overall reaction. The catalytic reaction processes of enzymes should reflect their protein structures. It was expected that some difference in a protein structure might be found between the yeast and the muscle enzyme. In this chapter, the results of fundamental properties of the yeast phosphoglucomutase protein and the chemical modification are described.

MATERIALS AND METHODS

Equilibrium centrifugation The molecular weight of yeast phosphoglucomutase was determined by means of the meniscus depletion method of Yphantis[25]. The equilibrium centrifugation experiments were carried out with a Beckman model E analytical ultracentrifuge equipped with a Rayleigh interference optical system.

Assay of the enzyme activity Unless otherwise speci-

fied, the reaction mixture contained 0.2 μmole of Gl-P, 5.0 nmoles of Gl,6-diP, 10 μmoles of Tris buffer (pH 7.5) and 10 μg of bovine serum albumin in a final volume of 1.0 ml. After the incubation at 25° for 10 minutes, the reaction was initiated by the addition of 5 μl of the enzyme solution. The reaction was terminated by the addition of 2.5 ml of 1M H_2SO_4. Acid-labile phosphate was determined by means of the method of Bartlett[26].

Reagents Crystalline bovine serum albumin, norleucine and cysteic acid were purchased from Sigma, and Gl-P and Gl,6-diP from Boehringer Mannheim. For the experiments involving substrate titration of the enzyme modified with ANS, Gl-P was purified chromatographically by means of Ray's method[9]. ANS was a commercial product. PCMB was used after purification and NBS was recrystallized from water.

Yeast phosphoglucomutase was crystallized as described in Chapter I.

Protein determination The turbidimetric method[27,28] was used to relate protein concentration to absorbance of the enzyme at 280 nm. The crystalline bovine serum albumin was used for the standard.

Amino acid analysis The enzyme was hydrolyzed at 110° for 22 or 70 hours in 6M HCl. Amino acid analyses

were performed with a Yanagimoto amino acid autoanalyzer LC-5S. For determination of the half-cystine content, a performic acid oxidation was carried out as described by Hirs[29].

The tryptophan content was determined from the absorbance at 280 nm and 294.4 nm of the enzyme in 0.1 M NaOH[30].

NBS oxidation An absorbance difference at 280 nm per mole of tryptophan oxidized by NBS, 4×10^3 (see ref.31), was used both in acetate and in urea.

PCMB titration The titrations of sulfhydryl groups of the enzyme were carried out with PCMB[32]. The value of modified SH-groups per mole of the enzyme was not calculated from absorbance change at 250 nm, but from the end-point estimated graphically (see Fig.15 and 16).

Fluorimetric titration with ANS[33] Fluorescence intensity (at 470 nm) of ANS bound by the enzyme was measured with a Hitachi fluorescence spectrophotometer, model MPF-2A. The excitation wavelength was 400 nm. Although fluorescence of ANS bound by the enzyme was maximally excited at 370 nm, it was excited at 400 nm in order to decrease the effect of quenching by absorption. The following equation was used for determination of the number, N, of ligand bound by the enzyme and the dissoci-

ation constant, K.

$$\frac{[S]}{\alpha} = \frac{1}{1-\alpha} K + N[E]$$

Where [S] and [E] represent the initial concentration of the ligand and that of the enzyme, respectively. The value of α is defined as $x/N[E]$, where x is the concentration of the sites of the enzyme bound by the ligand. K and N can be obtained from the plot of $[S]/\alpha$ *versus* $1/1-\alpha$.

RESULTS

Molecular weight

The plot of the logarithm of fringe displacement *versus* $(\text{radius})^2$ was linear as shown in Figure 14. The molecular weight was calculated from the slope of this plot. The partial specific volume of the enzyme, 0.75, was used for the calculation. The average of triplicate measurements is presented in Table IV. These data give a molecular weight of 69,500. This value was close to that of the enzymes from other origins[10-12,34].

Molecular extinction coefficient of the enzyme

The molecular extinction coefficient at 280 nm, 8.2 x $10^7 cm^2$/mole, was determined by means of the turbidimetric method.

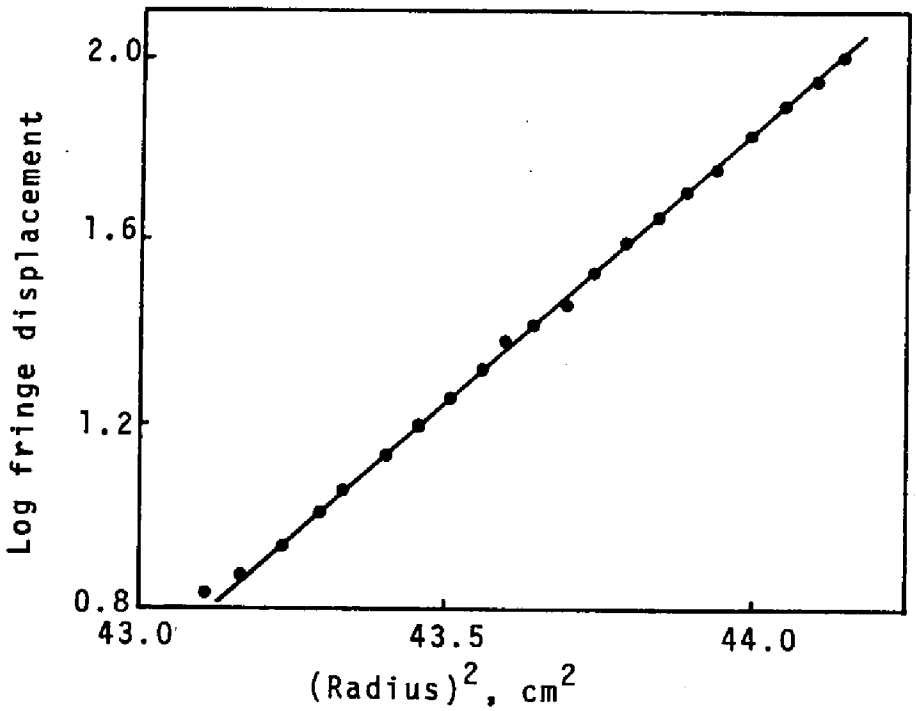

Figure 14 Molecular Weight of Yeast Phosphoglucomutase

The centrifugation was carried out at 10° with the rotor speed of 26,000 rev/min. The concentration of the enzyme was 0.035 %.

TABLE IV MOLECULAR WEIGHT DETERMINATION

The concentrations of the enzyme were 0.017, 0.035 and 0.05 % for each speed. Ionic strength was adjusted with NaCl to 0.1. Temperature was 10°.

Speed (rev/min)	Molecular weight
26,000	70,100
28,000	68,800
Average	69,500

Amino acid composition

The amino acid composition of the enzyme did not exhibit large differences from the compositions of the enzymes from rabbit muscle[35] and *E.coli*[10], except that the amounts of serine and tyrosine were relatively large, and those of methionine and arginine relatively small.

TABLE V AMINO ACID COMPOSITION OF YEAST PHOSPHOGLUCOMUTASE

Amino acid	Residues per mole of the enzyme	Amino acid	Residues per mole of the enzyme
Aspartic acid	68	Leucine	36
Threonine	33	Tyrosine	23
Serine	35	Phenylalanine	26
Glutamic acid	52	NH_3	77
Proline	26	Lysine	42
Glycine	50	Histidine	9
Alanine	40	Arginine	15
Valine	33	Half-cystine*	6
Methionine	3	Tryptophan**	7
Isoleucine	39		

*Estimated as cysteic acid.
**Estimated spectrophotometrically.

The tryptophan and tyrosine contents of the enzyme, as determined spectrophotometrically, were 7.2 and 25.9, respectively. The tyrosine contents determined by this method agreed approximately with the data from amino acid analysis.

PCMB titration

Figure 15 shows that 3.0 nmoles of the native enzyme are saturated with 12.3 nmoles of PCMB. This indicates that four sulfhydryl groups exist on the surface of the native enzyme.

The activity of the enzyme was not fully suppressed by PCMB. The sulfhydryl groups are, therefore, considered not to form the active center of the enzyme.

The titration of the enzyme was also performed in 5 M urea. About five sulfhydryl groups of the urea-denatured enzyme were modified with PCMB as shown in Figure 16.

It was suggested from the results of amino acid analysis and PCMB titration that four sulfhydryl groups existed on the surface of the enzyme, and that one or two were located inside the enzyme molecule.

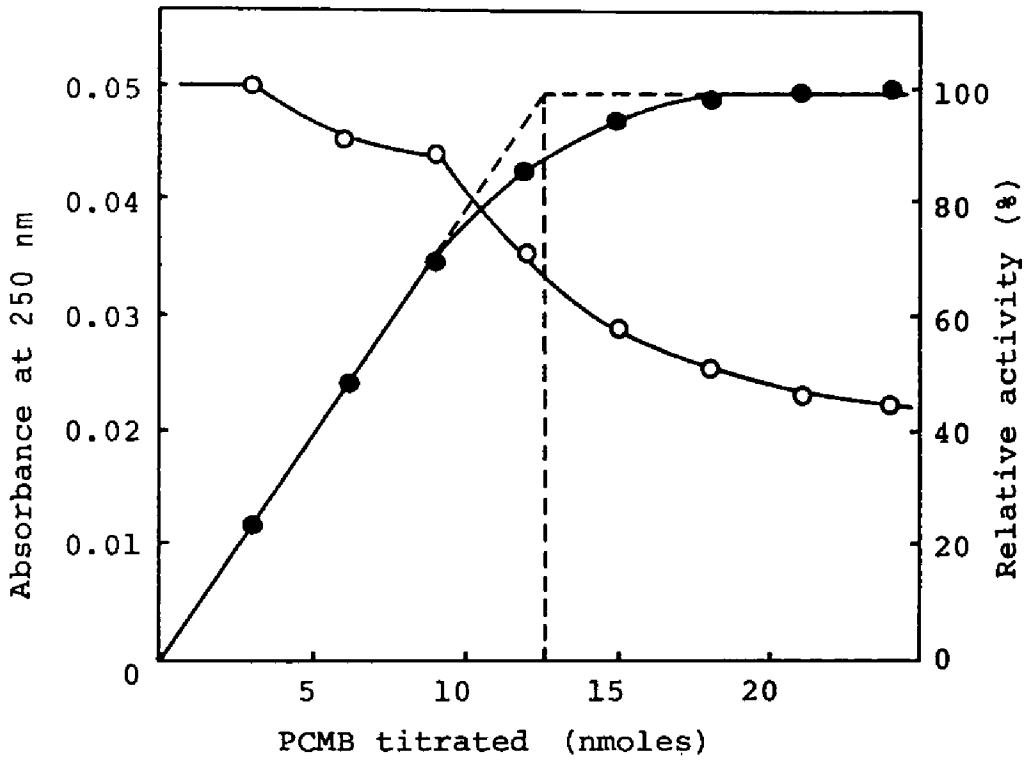

Figure 15 PCMB Titration of Yeast Phosphoglucomutase

The titration was carried out in 10 mM Tris buffer (pH 7.5) with 3.0 nmoles of the enzyme. After the incubation of the enzyme and PCMB for 20 minutes, the absorbance at 250 nm was recorded. Activity was determined by assaying 5 µl of the enzyme solution which had been removed from the cuvette.

Symbols : ● , absorbance at 250 nm ; o , relative activity.

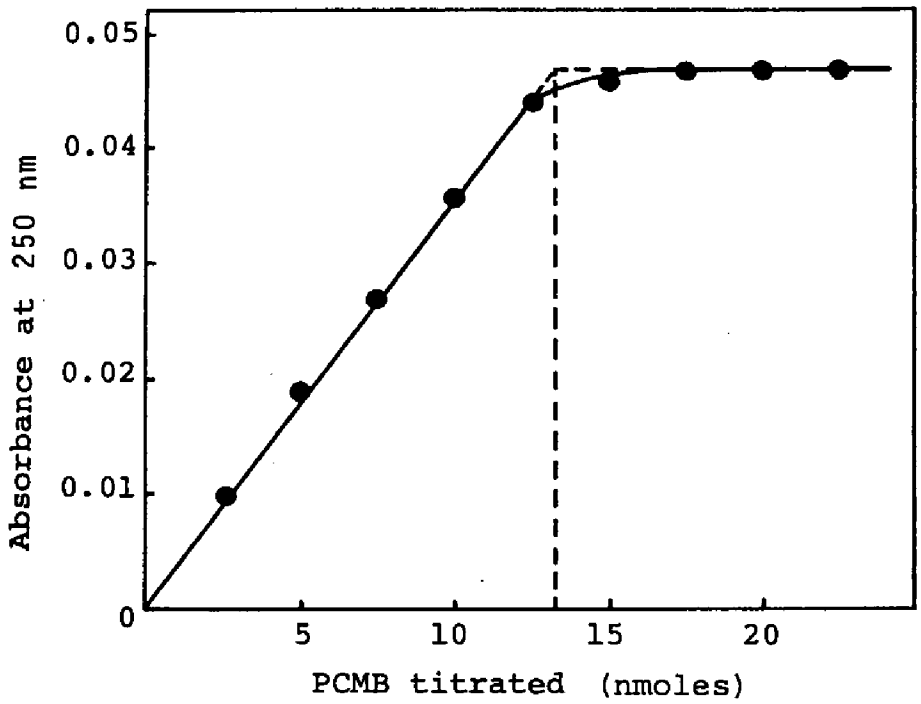

Figure 16 PCMB Titration of the Urea-Denatured Enzyme

The titration was performed in 5 M urea (in 10 mM Tris buffer (pH 7.5)) with 2.68 nmoles of the enzyme.

NBS oxidation

Figure 17 shows that seven tryptophan residues of the native enzyme are oxidized by NBS. Seven tryptophan residues of the urea-denatured (5 M) enzyme were also oxidized by NBS. These results coincided well with the values obtained from the absorbance of the enzyme

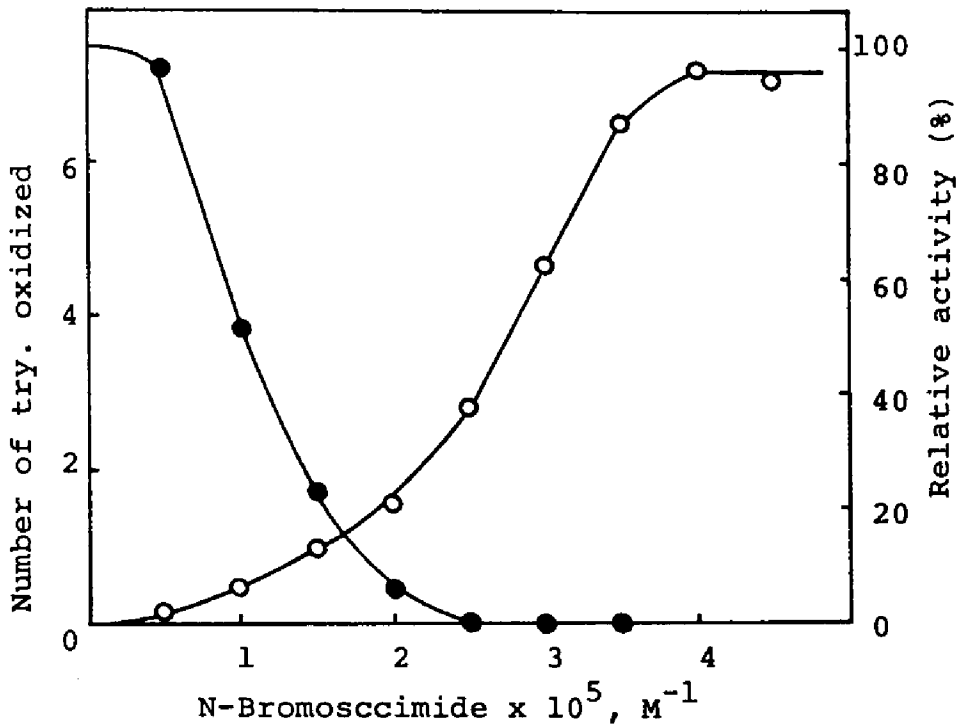

Figure 17 NBS Oxidation of Yeast Phosphoglucomutase

The oxidation was performed in 50 mM acetate buffer (pH 4.0). The concentration of the enzyme was 1.12 μM. Activity was determined by assaying 5 μl of the enzyme solution which had been removed from the cuvette.

Symbols : ○ , moles of tryptophan oxidized per mole of the enzyme ; ● , relative activity.

in 0.1 M NaOH and suggested that all the tryptophan residues existed on the surface of the enzyme.

Figure 17 also shows that the enzyme is fully inactivated by NBS and that the activity is decreased to about

15 % of that of the native enzyme by oxidation of one tryptophan residue per mole of the enzyme. The inactivation by NBS was approximately proportional to the decrease of the absorbance ; 5 and 48 % of the activity was lost on oxidation of 0.09 and 0.45 mole of tryptophan per mole of the enzyme, respectively. This suggested that the inactivation by NBS was due to oxidation of the tryptophan moiety of the enzyme and that one tryptophan residue of the enzyme played an important role in the activity of the enzyme.

The effect of Gl-P and Gl,6-diP on the inactivation by NBS was examined in pH 4.0 and in pH 7.5 (optimal pH for the enzyme activity). The substrate and the coenzyme were not able to protect the enzyme from inactivation by NBS. These results suggested that the tryptophan residue did not exist on the substrate or the coenzyme binding site of the enzyme. Indeed, the difference spectrum and the fluorescence spectrum change of the enzyme were not observed by the additions of the substrate and the coenzyme.

Titration with hydrophobic probe

The hydrophobic region of the enzyme was studied with a hydrophobic probe, ANS. Figure 18 and 19 show that the number of ANS units bound by the enzyme was not influ-

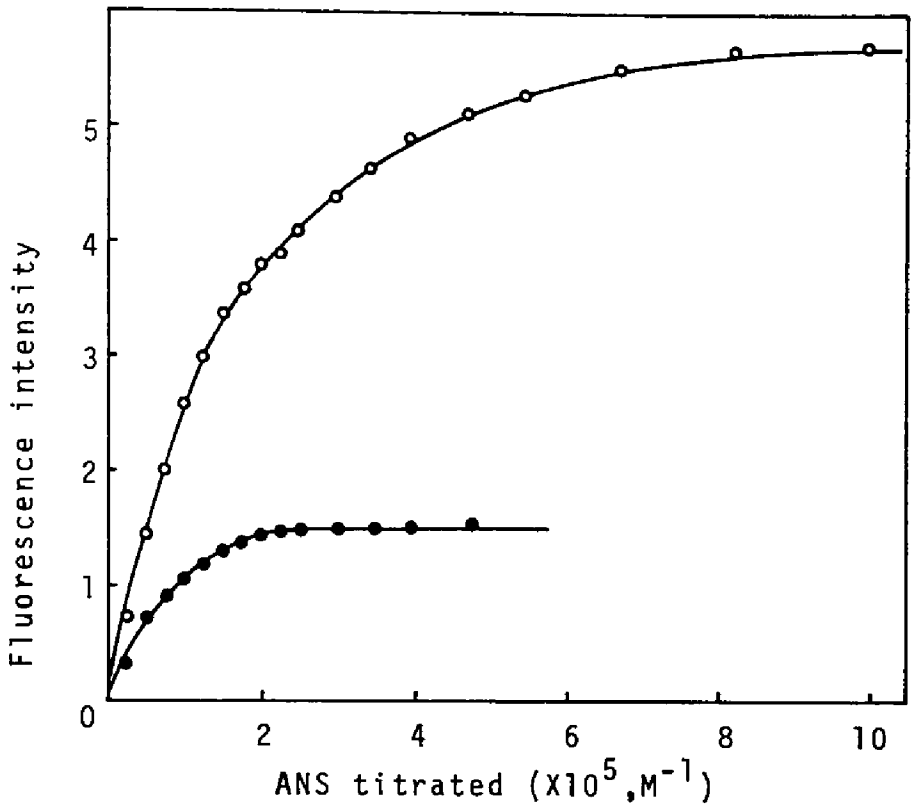

Figure 18 Fluorimetric Titration with ANS

The titrations were carried out in 10 mM Tris buffer (pH 7.5) by adding the solution of ANS to the enzyme solution in the presence of 1.0 mM Gl-P and 6.0 μM Gl,6-diP (●) or in the absence of the substrate and the coenzyme (o). The fluorescence intensities of ANS in the absence of the enzyme were subtracted from those of ANS in the presence of the enzyme.

enced by the addition of the substrate and the coenzyme, and that the dissociation constant was decreased by the substrate and the coenzyme. The dissociation constants,

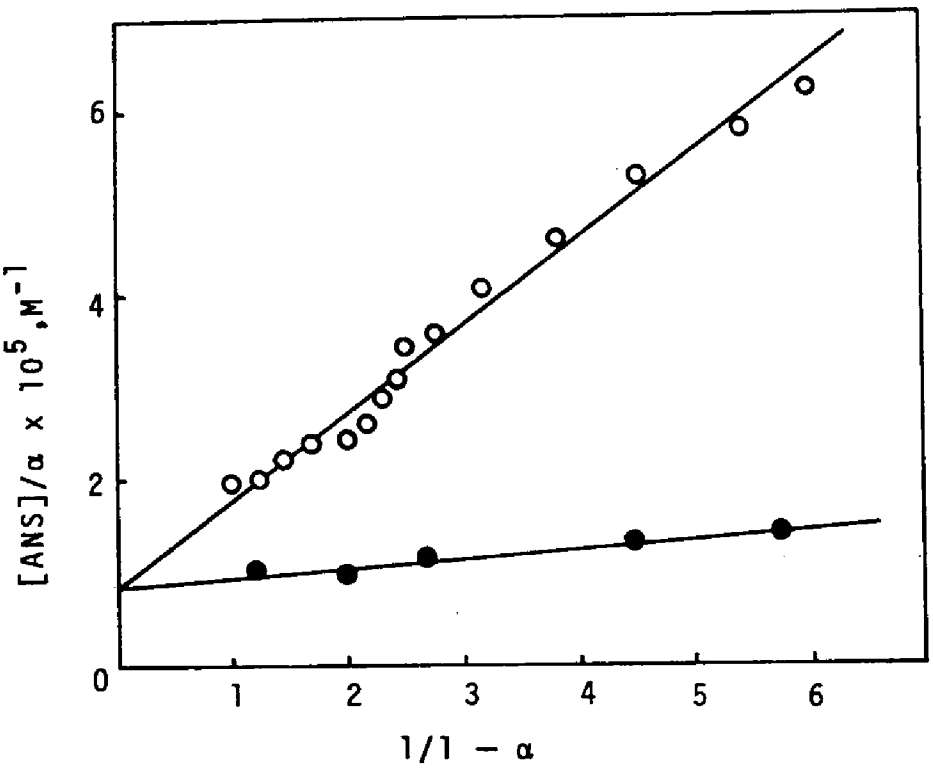

Figure 19 The Determination of N and K between ANS and the Enzyme

The values of α were obtained from the ratio of the individual increase to the maximum increase of fluorescence intensity in Fig.18.

Symbols : ● , the values in the presence of 1.0 mM Gl-P and 6.0 μM Gl,6-diP ; ○ , in the absence of the substrate and the coenzyme.

K, were 1.0 μM and 9.5 μM in the presence and in the absence of the substrate and the coenzyme, respectively. On the other hand, N was 8.5 under both conditions.

Figure 18 also shows that the fluorescence of ANS

bound by the enzyme was strongly quenched by the addition of the substrate and the coenzyme. The quenching by the substrate and the coenzyme was specific for the enzyme , since the fluorescence intensity of ANS bound by bovine serum albumin was not influenced by Gl-P and Gl,6-diP. These observations suggested that the state of a hydrophobic region of the enzyme was changed by the addition of the substrate and the coenzyme.

Binding studies of the substrate and the coenzyme

The quenching of ANS attached to the enzyme was also brought about by the individual addition of the substrate or the coenzyme. The enzyme saturated with ANS was titrated with Gl-P or Gl,6-diP. Figure 20 shows that the dissociation constants of Gl-P and Gl,6-diP to the enzyme are 14 μM and 0.16 μM, respectively, and that the number of moles of Gl,6-diP bound by the enzyme is 1.0. The number of moles of Gl-P bound by the enzyme was not able to be determined, since the dissociation constant of Gl-P to the enzyme was large compared with the concentration of the enzyme used. The K values obtained by titration experiments agreed tolerably with the Km value obtained by the kinetic studies (Km for Gl-P, 4.0 μM ; Km for Gl,6-diP, 0.14 μM).

The enzyme activity was not influenced by saturation

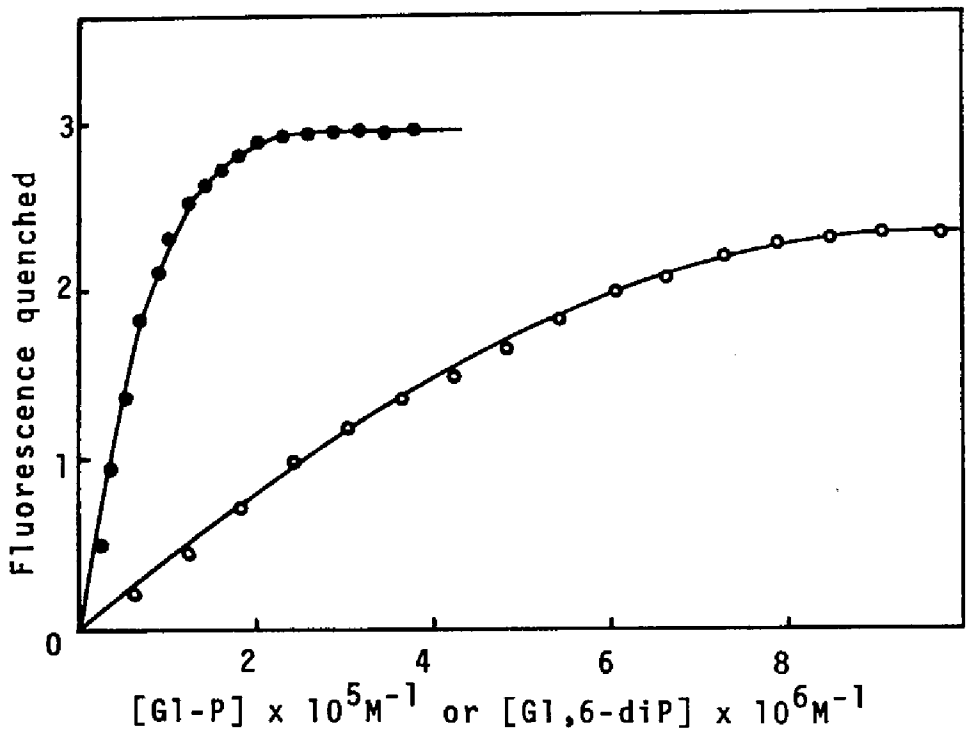

Figure 20 Titration of Gl-P and Gl,6-diP to the Enzyme Modified with ANS

Quenching of ANS bound by the enzyme was measured by the addition of Gl-P or Gl,6-diP. The enzyme was saturated with 0.1 mM ANS. The titrations of Gl-P (o) and Gl,6-diP (●) were carried out in Tris buffer (pH 7.5) with 0.82 μM of the enzyme.

of the enzyme with 0.1 mM of ANS in the presence of 0.2 mM Gl-P and 0.2 μM Gl,6-diP. This suggested that the affinities of the substrate and the coenzyme for the enzyme were not largely influenced by ANS.

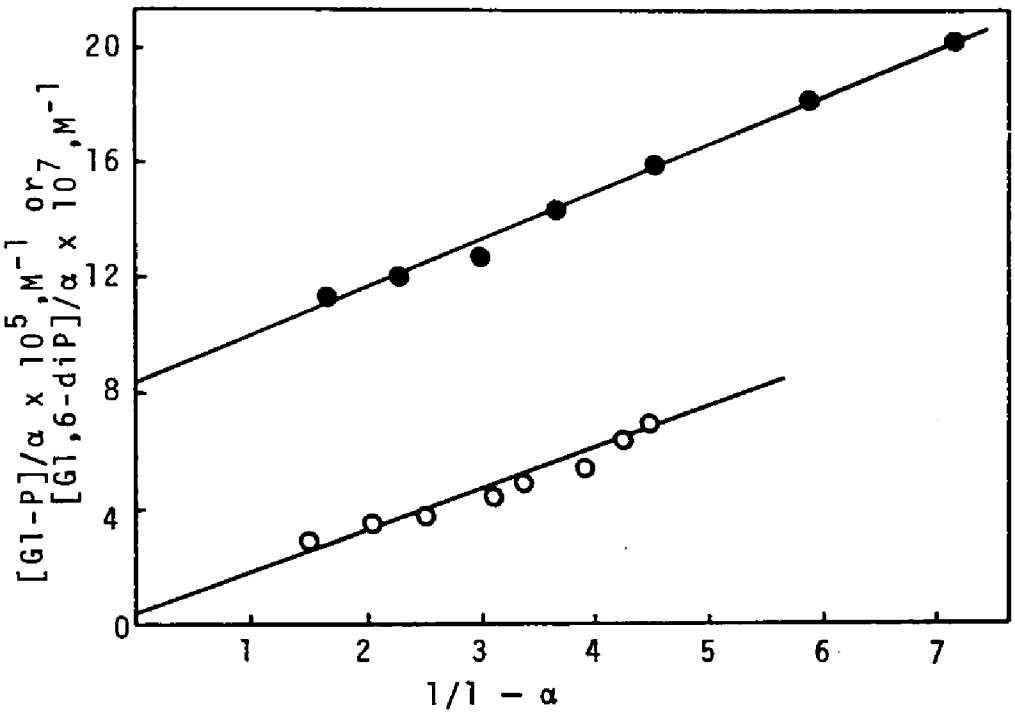

Figure 21 The Determinations of K and N of Gl-P and Gl,6-diP to the Enzyme

The experimental conditions are described in the legend of Fig.20. The values of α were obtained from the ratio of the individual decrease to the maximum decrease of fluorescence intensity in Fig.20.

Symbols : ○ , Gl-P ; ● , Gl,6-diP.

DISCUSSION

The results of amino acid analysis and molecular weight determinations of yeast phosphoglucomutase did not show particular characteristics compared with the rabbit muscle enzyme[34,35].

It was suggested that the tryptophan residue of the enzyme played an important role for the activity of the enzyme. However, the residue did not probably exist on the substrate and coenzyme binding sites of the enzyme. It may be supposed that the residue is required for a catalytic step in the enzyme reaction or for the maintenance of the highly ordered structure of the enzyme.

The quenching of ANS attached to the enzyme was individually induced by the substrate and the coenzyme. The dissociation constants of the substrate and the coenzyme to the enzyme agreed tolerably with the Km values obtained by kinetic experiments. This provided further evidence for a "sequential" mechanism of the yeast enzyme reaction. If the reaction of yeast enzyme proceeds *via* a "ping-pong", the enzyme must be exist in the dephospho-form, because the reaction did not proceed without the addition of the coenzyme. The substrate should not combine with the dephospho-enzyme in a "ping-pong" mecha-

nism, unless the substrate concentration is increased in order to bring about the substrate inhibition. In the yeast enzyme, the substrate inhibition constant was 1.0 mM. It was impossible that the inhibition constant was decreased to 14 μM by the binding of ANS to the enzyme, since the activity was not inhibited by ANS in the presence of 0.2 mM Gl-P and 0.2 μM Gl,6-diP. Thus the dissociation constant of the substrate to the enzyme measured by fluorimetric titration will not mean the substrate inhibition constant, but the Km value.

The "sequential" mechanism is divided into the terms of "random sequential" and "ordered sequential"[8]. Although the kinetic experiments had probably suggested the former, the latter had not fully been excluded. The results of fluorimetric titration also suggested a "random sequential" mechanism for a reaction of the yeast enzyme, since the substrate and the coenzyme were individually able to attach to the enzyme.

Chapter V Essential Metal Ion for the Yeast Phosphoglucomutase Reaction

In the previous chapter, yeast phosphoglucomutase protein did not show a particular characteristics compared with the rabbit muscle enzyme in the elucidation of the difference of a reaction sequence. The yeast enzyme was different from the muscle enzyme on the effects of a bivalent cation for the catalytic activity. It was reported that rabbit muscle phosphoglucomutase required Mg^{2+} for the catalytic activity and was stimulated by the simultaneous addition of chelating-agent and Mg^{2+} (see ref.3,36-38). On the other hand, the yeast enzyme was active without any addition of a bivalent cation to the reaction mixture, and was strongly inhibited by chelating-agents. The stimulation by the simultaneous addition of Mg^{2+} and chelating-agents was observed also in the yeast enzyme reaction. It was expected that the difference in the reaction mechanism between yeast and muscle enzyme was due to the difference in the requirement of a bivalent cation.

In this chapter, the inhibition and stimulation mechanisms of EDTA are described.

MATERIALS AND METHODS

Reagents ANS and the standard metal ion solutions (1.0 mg/ml $ZnCl_2$ and 1.0 mg/ml $MgCl_2$) were obtained commercially.

Glass-distilled water, which was obtained by a glass distillation of deionized water, was used in all experiments. Tris buffer (pH 7.5), citrate buffer (pH 5.3) were purified by passage through the Amberlite IR-120 columns which were previously equilibrated with each buffer. Gl-P, G6-P and Gl,6-diP were also purified with use of Amberlite IR-120 (H^+-form).

Crystalline yeast phosphoglucomutase was prepared as described in Chapter I. The concentration of the enzyme was determined on the basis of molecular extinction coefficient at 280 nm, $8.2 \times 10^7 cm^2$/mole as described in Chapter IV.

Assay of the enzyme activity The enzyme activity was measured as described in Chapter I except that the temperature was 25°.

Atomic absorption measurements The metal content of the enzyme was determined with Nippon Jarrell-Ash atomic absorption spectrophotometer (model AA-70) equipped with a Hitachi Recorder (model QPD-34). Absorption wave-

lengths were 213.86 nm and 285.21 nm on the measurements of zinc and magnesium, respectively. The standard metal solutions were used after dilution with the same buffer as the medium of the enzyme solution.

Fluorescence measurements Fluorescence intensity of ANS bound by the enzyme was measured with a Hitachi fluorescence spectrophotometer (model MPF-2A). The excitation and the emission wavelengths were 400 nm and 470 nm, respectively. The number of ANS bound by the enzyme was determined as described in Chapter IV.

RESULTS

Stimulatory and inhibitory effects of EDTA

When the reaction was started with the addition of the enzyme solved in 10 mM acetate buffer (pH 5.3), the enzyme activity was stimulated by lower concentrations of EDTA and was inhibited by higher concentrations of EDTA as shown in Figure 22. EDTA exerted two types of the effects on the yeast enzyme activity without any addition of a bivalent cation. It was also shown in Figure 22 that EDTA exerts only a stimulatory effect on the enzyme activity in the presence of Mg^{2+} or Mn^{2+}. A higher concentration of EDTA was required for the stimulation of

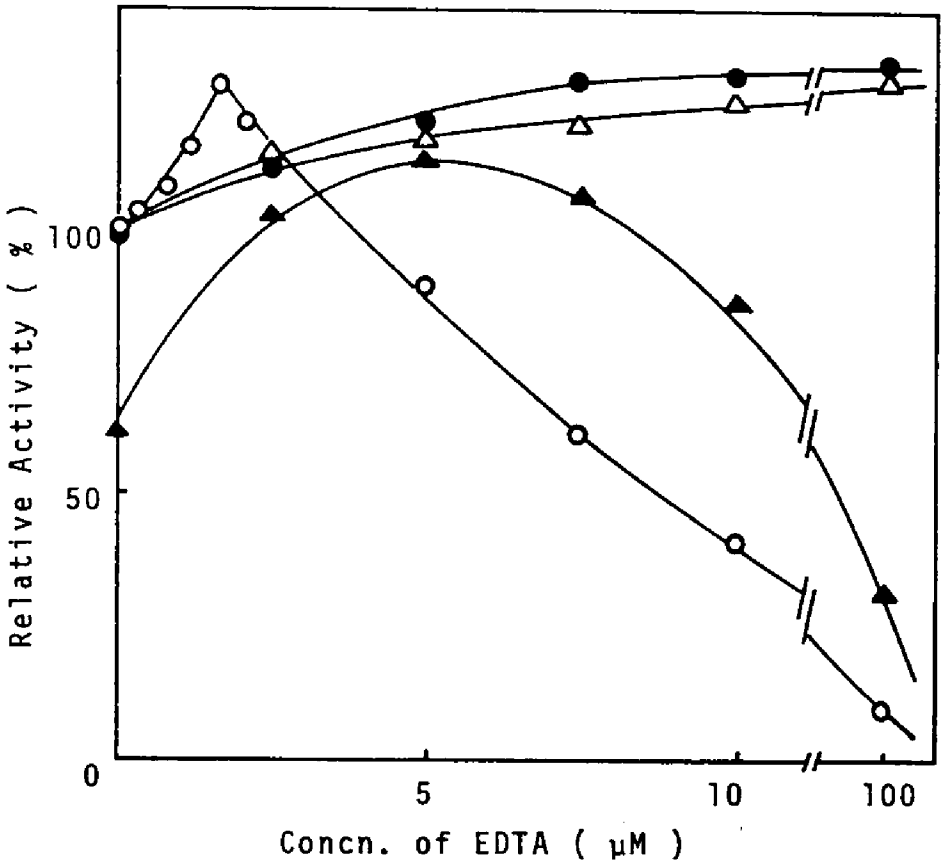

Figure 22 Effects of EDTA and Bivalent Cations

The reactions were started by the addition of the enzyme solved in 10 mM acetate buffer (pH 5.3).
Addition to the reaction mixture : ○ , none ; ● , 1.0 mM $MgCl_2$; △ , 0.50 mM $MnCl_2$; ▲ , 5.0 μM $ZnCl_2$.

the enzyme in the presence of Mg^{2+} or Mn^{2+} than in the absence of the bivalent cations, and the extents of the stimulation were identical under both conditions. These observations suggested that a bivalent cation was not

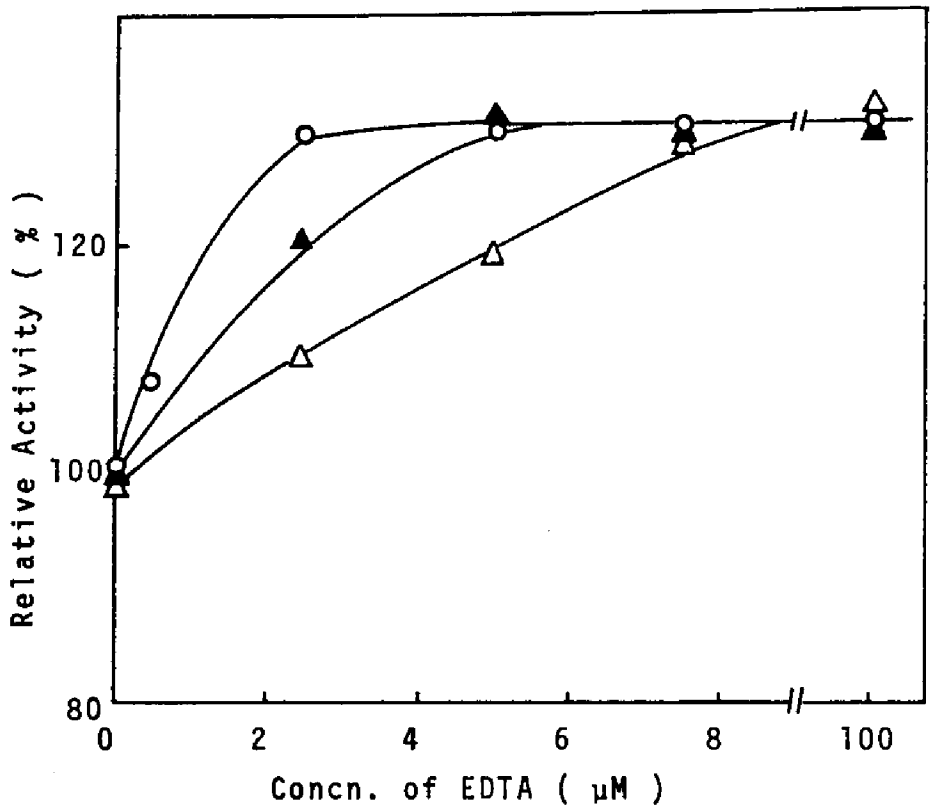

Figure 23 Effects of EDTA and Mg^{2+}

The reactions were started by the addition of the enzyme solved in 10 mM Tris buffer (pH 7.5).
Addition of $MgCl_2$ to the reaction mixture : ○ , none ; ▲ , 0.50 mM ; △ , 2.0 mM.

essential to the stimulation but protected the enzyme activity from the EDTA-inhibition by decreasing the concentration of free EDTA. Thus EDTA exerted a stimulatory effect in the presence of Zn^{2+}, which itself showed

an inhibitory effect.

It was assumed from these observations that the stimulation and the inhibition by EDTA occurred in a different site on the enzyme surface. This was supported by the observation under another experimental condition. When the reaction was started by the addition of the enzyme solved in 10 mM Tris buffer (pH 7.5), EDTA exerted only a stimulatory effect and the presence of Mg^{2+} lowered the effects of EDTA as shown in Figure 23. Under this condition, the enzyme activity was perfectly protected from the inhibition by EDTA without any addition of a bivalent cation.

The enzyme activity was decreased to 3-70% of the native enzyme activity by the preincubations of the enzyme with various bivalent cations (0.20 mM Co^{2+}, 20 μM Cu^{2+}, 20 μM Zn^{2+} and 2.0 μM Hg^{2+}) in Tris buffer (pH 7.5). The lowered enzyme activities were almost completely recovered by the addition of 0.1 mM EDTA to the reaction mixture with the exception of Hg^{2+}. This suggested that the stimulatory effect of EDTA was due to a removal of inhibitory metal(s) attached to the enzyme.

The formation of the enzyme-EDTA complex

It was demonstrated by the EDTA-inhibition that a bivalent cation was essential to the yeast phosphogluco-

mutase reaction. The yeast enzyme was active without any addition of a bivalent cation. These effects suggested that the essential bivalent cation was strongly bound by the yeast enzyme.

The mechanism of EDTA-inhibition was examined. The enzyme was dialyzed against 10 mM citrate buffer (pH 5.3) containing 0.1 mM EDTA which almost completely inactivate the enzyme activity (see Fig.22). However, the activity was perfectly recovered by lowering the EDTA concentration with a dilution of the enzyme solution or by the removal of EDTA with the passage through the Sephadex G-50 column which was equilibrated with 10 mM Tris buffer (pH 7.5). These observations suggested that the EDTA-inhibition was not due to the removal of the essential metal ion from the enzyme, but to a formation of the enzyme-metal-EDTA complex.

The yeast enzyme activity was not inhibited by EDTA when the reaction was started by the addition of the enzyme solved in the neutral buffer, as shown in Figure 23. However, the activity was decreased with the proceeding of time of preincubation with EDTA in the neutral buffer as shown in Figure 24. The extent of the inhibition was not changed if higher concentrations of EDTA than those in the preincubation mixture were added to the

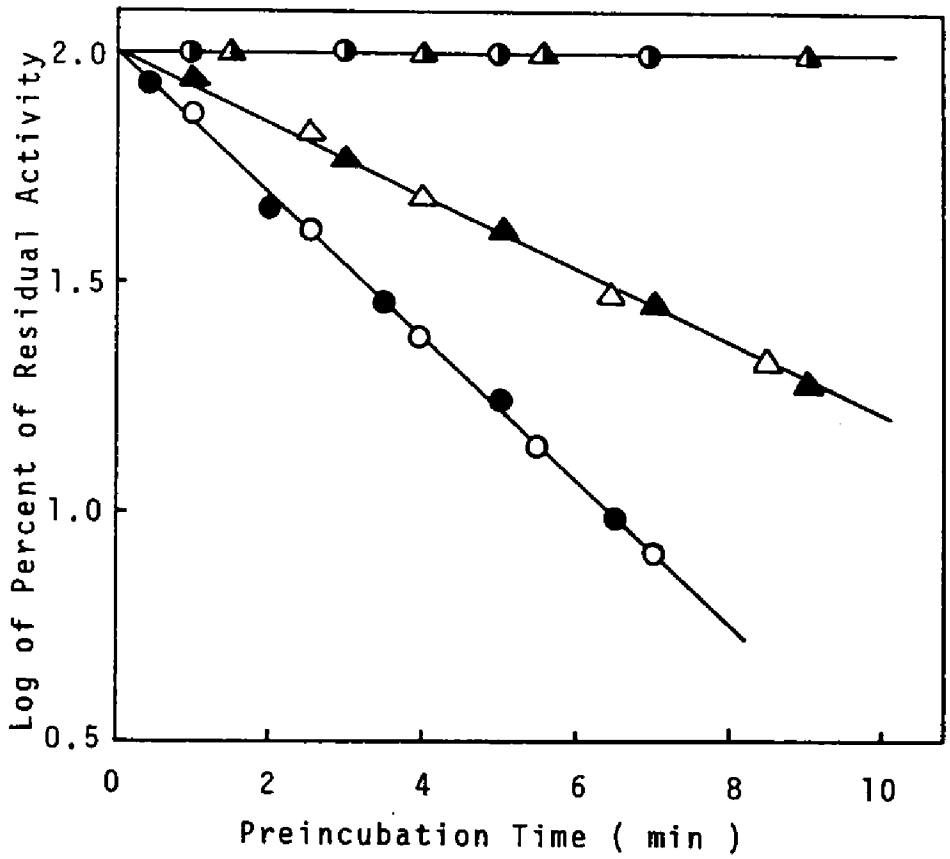

Figure 24 The Time Dependent Inhibition of EDTA

The enzyme (0.50 μM) was preincubated with EDTA in 10 mM Tris buffer (pH 7.5) at 25°. The reactions were started by the addition of 40 μl of the enzyme solution to 0.36 ml of the mutase reaction mixture. The mutase reaction were carried out as described in the text. The ordinate represents the logarithm of percent of the residual activity to the activity without preincubation.

Addition of EDTA to the preincubation mixture : 10 μM (○ , ● , ◐), 5.0 μM (△ , ▲ , ▲).

Addition of EDTA to the mutase reaction mixture : none (◐ , ▲), 20 μM (● , ▲), 0.20 mM (○ , △).

mutase reaction mixture. These observations suggest that the enzyme-EDTA complex is formed in the preincubation, and that EDTA in the mutase reaction prevents the dissociation of the enzyme-EDTA complex formed in the preincubation.

It is clear from Figure 24 that the formation of the enzyme-EDTA complex follows apparent first-order kinetics. This result is expected since the concentrations of EDTA are large compared with the enzyme concentration. The apparent first-order rate constant in the presence of 10 μM EDTA is twice that in 5.0 μM EDTA (Fig.24).

The binding site of EDTA

It was observed in Figure 23 and 24 that the formation of the enzyme-EDTA complex did not occur in the mutase reaction mixture. The EDTA-inhibition did not occure under the same conditions as in Figure 24 with addition of 2.0 mM Gl-P and 12.5 μM Gl,6-diP to the preincubation mixture. EDTA might bind to the enzyme competitively with the substrate or the coenzyme. The protective effect of various concentrations of the substrates and the coenzyme were examined to determine the dissociation constants of the binary complexes of the enzyme and the substrates or the coenzyme. Figure 25 shows that the apparent first-order reaction constant was

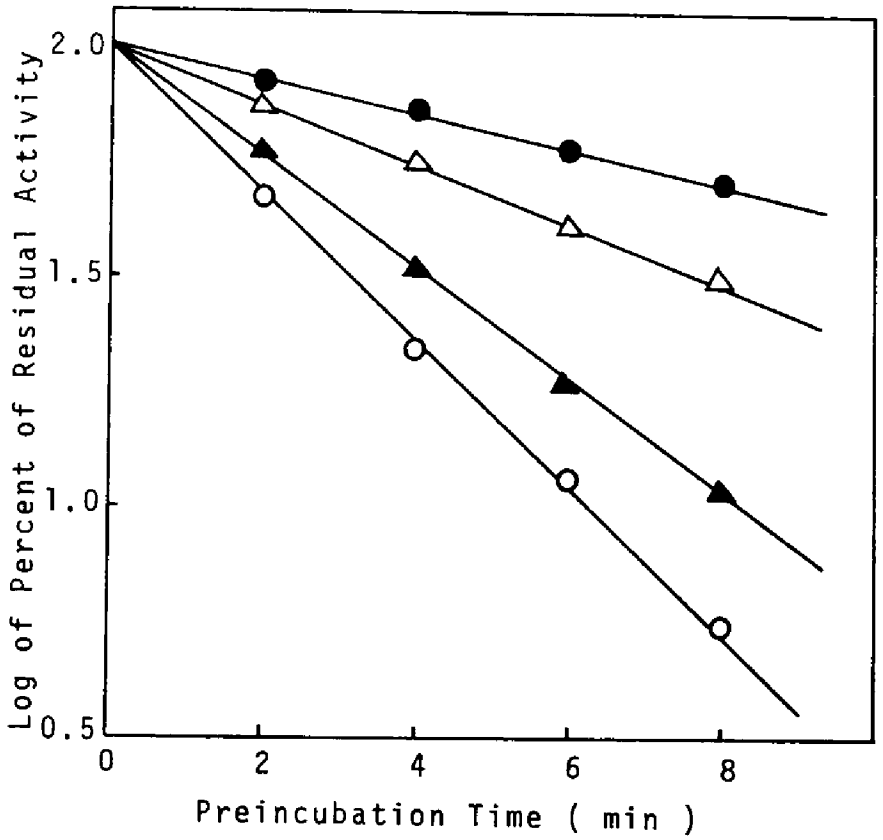

Figure 25 Protective Effects of the Substrates and the Coenzyme from the EDTA-Inhibition

The enzyme was preincubated with 10 μM EDTA in the presence or the absence of the substrate and the coenzyme. The mutase reaction mixture contained 20 μM EDTA. Other conditions for the preincubation and the mutase reaction were same as in Fig.24.

Addition to the preincubation mixture : ○ , none ; △ , 0.60 mM Gl-P ; ▲ , 0.20 mM G6-P ; ● , 0.80 μM Gl,6-diP.

decreased by the addition of the substrate or the coenzyme to the preincubation mixture.

In the absence of the ligand (the substrate or the coenzyme), the apparent first-order rate constant, k, is:

$$\frac{d[EI]}{dt} = k([E]_0 - [EI])$$

Where E, I and EI represent the enzyme, EDTA and the enzyme-EDTA complex, respectively. In the presence of the ligand, following equations are obtained on the assumption that the protective effect of the ligand is due to a competition with EDTA.

$$\frac{d[EI]}{dt} = k([E]_0 - [EI] - [EL]),$$

$$\frac{([E]_0 - [EI] - [EL])[L]}{[EL]} = Ks$$

Where L and Ks represent the ligand and the dissociation constant of the enzyme-ligand complex, respectively. Thus the first-order reaction in the presence of the ligand is shown as follows.

$$\frac{d[EI]}{dt} = k \cdot \frac{Ks}{Ks + [L]} ([E]_0 - [EI])$$

The apparent first order rate constant in the presence of the ligand, k', is :

$$k' = k \cdot \frac{Ks}{Ks + [L]}$$

TABLE VI DISSOCIATION CONSTANTS OBTAINED FROM THE PROTECTIVE EFFECTS FROM THE EDTA-INHIBITION

The enzyme activity was assayed after the incubation at 25° in 10 mM Tris buffer (pH 7.5) with 10 μM EDTA in the presence or the absence of the ligands. The values of kinetic experiments are the data in Chapter II.

Ligands	Protection Experiments				Kinetics	
	Concn.	k'/k	Ks		Km	Ki
Gl-P	1.0 mM	0.23	0.3 mM	Av. 0.5 mM	4.0 μM	1.0 mM
	0.6 mM	0.41	0.4 mM			
	0.1 mM	0.87	0.7 mM			
G6-P	1.1 mM	0.29	0.5 mM	Av. 0.5 mM	—	—
	0.2 mM	0.71	0.5 mM			
Gl,6-diP	10 μM	0.02	0.2 μM	Av. 0.2 μM	0.14 μM	—
	1.0 μM	0.16	0.2 μM			
	0.8 μM	0.23	0.2 μM			

This indicates that the Ks value can be calculated from the values of k and k' obtained experimentally. Table VI shows the Ks values for Gl-P, G6-P and Gl,6-diP obtained from this treatment. The important result in the table was that the Ks value for Gl,6-diP coincided

well with the Km value for the coenzyme obtained from kinetic experiments in Chapter II. The Ks value for Gl-P, however, did not agree with the Km value for the subatrate, but with the substrate inhibition constant, Ki. In the yeast phosphoglucomutase reaction, the substrate inhibition occurred with the binding of the substrate to the coenzyme binding site as shown in Chapter II. These observations suggest that the EDTA-inhibition may be due to binding to the coenzyme binding site.

Metal content of yeast phosphoglucomutase

Metal content of yeast phosphoglucomutase was assayed with atomic absorption spectrophotometry. The enzyme solution was dialyzed overnight against 10 mM citrate buffer (pH 5.3) containing 0.1 mM EDTA and was passed through a Sephadex G-50 column equilibrated with the same buffer to remove extraneous metal ions. Table VII represents that yeast phosphoglucomutase contains 0.87 mole of zinc, whereas magnesium content, the essential metal for the muscle enzyme reaction, is negligibly low.

Yeast phosphoglucomutase in the neutral and the acid conditions

The difference between the acid and the neutral enzyme shown in Figure 22 and 23 was studied with a hydrophobic probe, ANS. It was shown in the previous chapter

TABLE VII METAL CONTENT OF YEAST PHOSPHOGLUCOMUTASE

The enzyme solution was dialyzed overnight against 10 mM citrate buffer (pH 5.3) containing 0.1 mM EDTA, and was subjected to gel filtration with Sephadex G-50 column (1.6 x 20 cm) which was equilibrated with the same buffer.

Metal	Observed metal (μM)	Concn. of the enzyme (μM)	Mole of metal per mole of the enzyme	
Zinc	0.93	1.10	0.84	Av. 0.87
	1.15	1.30	0.88	
	1.60	1.80	0.89	
Magnesium	$<$0.05	0.80	$<$0.06	
		1.40		

that about eight ANS molecules were bound by the enzyme in the neutral buffer. The number was identical in the acid buffer, and was not changed by the addition of EDTA. However, the fluorescence intensity of ANS bound by the enzyme was quenched time-dependently by the addition of EDTA. Figure 26 shows that the quenching proceeds as an apparent first-order reaction. In neutral solution, the half-life was 1.8 minutes. The half-life obtained

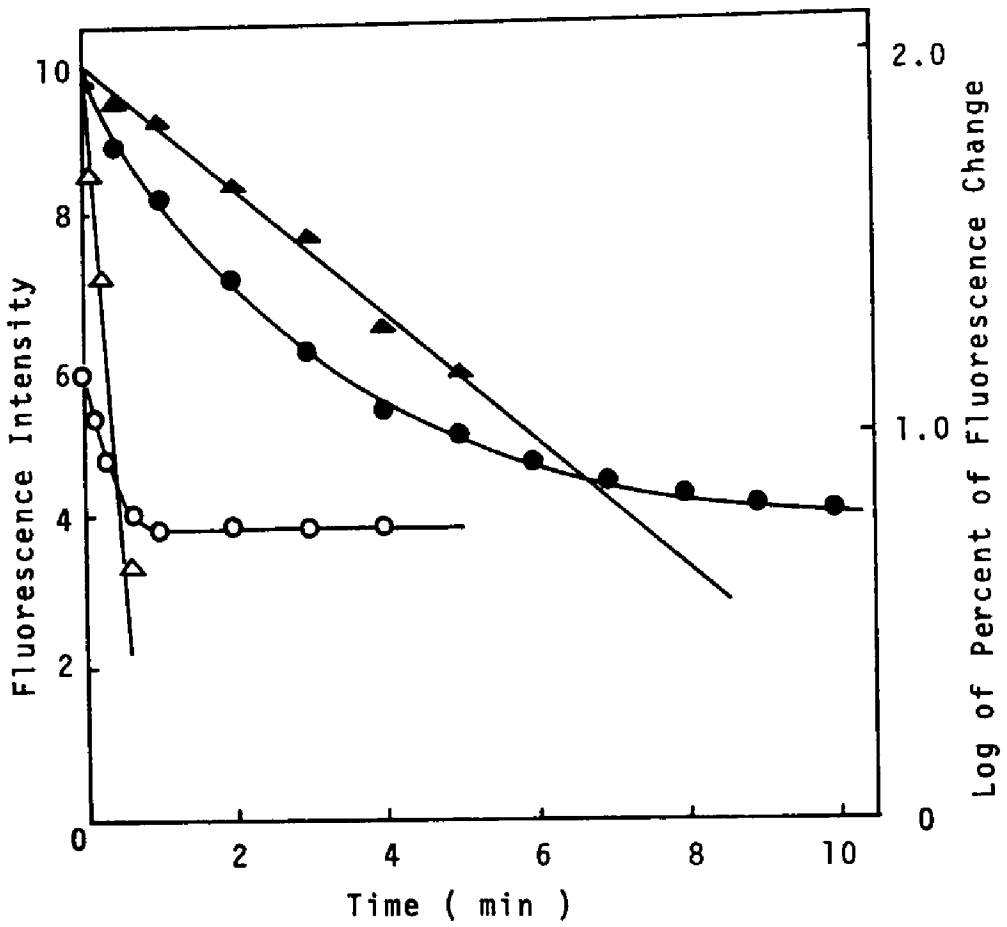

Figure 26 The Time-Dependent Quenching of the Fluorescence of ANS Induced by EDTA

To 2.0 ml of the enzyme solution containing 0.20 mM ANS and 20 mM ammonium-sulfate in a cuvette, 10 µl of 2.0 mM EDTA was added. The decrease of the fluorescence of ANS bound by the enzyme was measured in 10 mM acetate buffer (pH 5.3) or in Tris buffer (pH 7.5). The values in the right ordinate were calculated from the ratio of each decrease to the maximum decrease of the fluorescence intensity (△ , in acetate ; ▲ , in Tris).

Symbols : ○ , fluorescence in acetate buffer ; ● , in Tris buffer.

from the inhibitory effect of the same concentration of EDTA was 1.9 minutes as shown in Figure 24. This agreement suggests that the state of the hydrophobic region of the enzyme molecule is changed by the binding of EDTA. On the other hand, the half-life in ANS-quenching was about 10 seconds in acid solution. The enzyme-EDTA complex was formed in acid solution with a speed of one magnitude faster than in neutral solution.

DISCUSSION

It was indicated that intrinsic metal was essential for the yeast phosphoglucomutase reaction, and that the EDTA-inhibition was due to the formation of the enzyme-metal-EDTA complex. About one mole of zinc per mole of the enzyme, which was not removed by the treatment with EDTA, was observed by atomic absorption measurements. This intrinsic zinc may not be conclusively shown to be essential for the catalytic activity of the yeast enzyme, since the criteria proposed by Vallee[39] for the identification of a metalloenzyme were not always fulfilled. However, it is unlikely that significant amounts of transition metals are contained by yeast phosphoglucomutase, since the yeast enzyme did not show any absorption at

visible wavelengths. The various enzymes[39] are known to be zinc-metalloenzyme. It was reported that such enzymes from yeast as alcohol dehydrogenase[39], phosphomannose isomerase[40], fructose 1,6-diphosphate aldolase[41] and pyruvate carboxylase[42] were zinc-metalloenzyme. As pointed out by Scrutton et al.[42], metalloenzymes from yeast typically seem to contain zinc as the intrinsic metal ion. It was shown in Chapter II and IV that the reaction of yeast phosphoglucomutase proceeded *via* "random sequential" pathway. Thus the binding sites of the substrate and the coenzyme are considered to be different on the yeast enzyme surface. The protective effect of the coenzyme from the EDTA-inhibition suggested that the intrinsic metal existed on the coenzyme binding site. In Chapter IV, it was shown that one mole of the coenzyme was bound by one mole of the enzyme. This value agreed with the zinc content of the enzyme. These observations support the thesis that the intrinsic zinc existed on the coenzyme binding site.

The difference between the yeast and the muscle enzyme in the stimulation by chelating-agents can be elucidated by the facts that the yeast enzyme contained the intrinsic metal and the muscle enzyme required the extrinsic Mg^{2+} (see ref.3,36-38). The simultaneous

additions of a chelating-agent and Mg^{2+} were required for the activation of the muscle enzyme[3,36-38]. It was indicated by Milstein that the role of a chelating-agent for the activation of the muscle enzyme was to bind inhibitory heavy-metal ions and hence to aid the addition of Mg^{2+} to the enzyme[16,17]. Thus cysteine was the best activator and EDTA was the poor, since the former has a high affinity for heavy metals and a low affinity for Mg^{2+}, and the latter, a high affinity for both metal ions[16,17]. However, the yeast enzyme activity was able to be stimulated by the addition of EDTA independent of a bivalent cation. This suggested that EDTA-stimulation was due to the removal of inhibitory metal(s) attached to the different site(s) from that bound by the essential intrinsic metal.

The difference between Figure 22 and 23 may be elucidated as follows. The binding of EDTA to the yeast enzyme in the neutral solution was relatively slow and was reduced by the presence of the coenzyme. Thus the enzyme activity is not inhibited by EDTA, when the reaction is started by the addition of the enzyme solution dissolved in the neutral buffer. On the other hand, in the acid solution the enzyme was bound by EDTA in a relatively high speed. This difference between in the

neutral and in the acid solution is considered to be due to the difference in the state of the enzyme, since EDTA has a higher affinity to free metal ions in higher pH than in lower pH. The EDTA-inhibition in Figure 22 may be due to the binding of EDTA to the acid-state enzyme within the conversion of the acid-state to the neutral-state of the enzyme.

It was reported by Ray et al.[43-45] that in the muscle enzyme reaction Ni^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+} were able to substitute for Mg^{2+} and that the bivalent cations induced the conformational change in the enzyme molecule. They suggested from the values of the dissociation constants of the metal-enzyme complexes that muscle phosphoglucomutase might be classed as a "metalloenzyme" in the case of zinc and as a "metal-activated enzyme" in magnesium[43]. It was also demonstrated that zinc-enzyme exerted only 0.3 % activity of magnesium-enzyme, and Zn^{2+} acted as an inhibitor in the muscle enzyme reaction[43]. However, the specific activity of yeast phosphoglucomutase did not show a large difference from that of the muscle enzyme saturated with Mg^{2+} as shown in Chapter I. These differences in requirements of metal ion between the yeast and the muscle phosphoglucomutase seem to be important for understanding of the reaction

sequences of the mutases.

In the phosphoglucomutase reaction, a phosphate group of the coenzyme must be hydrolyzed. The hydrolyzed phosphate group must be directly transferred to the substrate in the "sequential" pathway of the yeast enzyme, whereas the phosphate group must be transferred to the enzyme to form the stable enzyme-phosphate complex in the "ping-pong" pathway of the muscle enzyme. It was shown in the muscle phosphoglucomutase reaction that the order of addition of Mg^{2+} and Gl-P to the phospho-enzyme, and of Mg^{2+} and Gl,6-diP to the dephospho-enzyme was random type[46]. According to the results of the report[46], the binding of Mg^{2+} to the muscle enzyme did not affected the binding of the substrate to the enzyme and slightly facilitated that of the coenzyme. These suggested that Mg^{2+} was not required to the binding of the substrate and the coenzyme but to the catalytic step of the muscle mutase reaction. However, it was suggested in this chapter that the intrinsic zinc played an important role for the binding of the coenzyme to the yeast enzyme.

It is considered that the bivalent cations play one of the important role in the reaction sequences of phosphoglucomutases.

Summary

Phosphoglucomutase was crystallized from baker's yeast. It was shown by the analytical ultracentrifugation and the electrophoretic analysis with cellulose-acetate membrane that this enzyme preparation was homogeneous.

The reaction mechanism of this enzyme was studied by the kinetic experiments. It was shown that the reaction of yeast phosphoglucomutase proceeded *via* a "sequential" pathway. The results of the fluorimetric titration with ANS suggested that the substrate and the coenzyme were able to bind to the yeast enzyme independently. The reaction mechanism of yeast phosphoglucomutase is, therefore, considered to be a "random sequential" type.

It was found that Fl,6-diP was able to act as a coenzyme of yeast phosphoglucomutase. The mechanism of the coenzymatic activity of Fl,6-diP was studied. It was indicated that in the Fl,6-diP dependent reaction that Gl,6-diP was formed by the phosphate-transfer of Fl,6-diP to Gl-P in the first step, and in the second step the original mutase reaction occurred in the presence

of Gl,6-diP. The reaction of the first step was reasonably interpreted by "sequential" pathway of yeast phosphoglucomutase.

The reaction mechanism of the yeast enzyme differed from the muscle enzyme, since the reaction of the muscle enzyme has been reported to proceed *via* a "ping-pong" pathway. The properties of the yeast enzyme protein were studied to clarify the difference in the reaction mechanism of phosphoglucomutases. The results of amino acid analysis and molecular weight determinations of yeast phosphoglucomutase did not show particular characteristics compared with the muscle enzyme. The states of amino acid residues of the enzyme were studied by means of PCMB titration, NBS oxidation and the titration of hydrophobic probe, ANS. It was suggested by the NBS oxidation that one tryptophan residue played an important role for the activity of the enzyme. It was demonstrated from the fluorescence intensity of ANS attached to the enzyme that the states of hydrophobic regions of the enzyme were changed by both the substrate and the coenzyme.

The major difference between the yeast and the muscle enzyme protein was found in the essential metals for the catalytic activities of the enzymes. It was reported that the addition of the extrinsic magnesium ion was

required for the muscle phosphoglucomutase reaction. However, it was suggested from the results of the protective effect of coenzyme from the EDTA-inhibition and of atomic absorption spectrophotometry that the intrinsic zinc existed on the coenzyme binding site of yeast phosphoglucomutase. These results suggested that bivalent cations played one of the important role in a determination of the reaction mechanism of phosphoglucomutase.

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